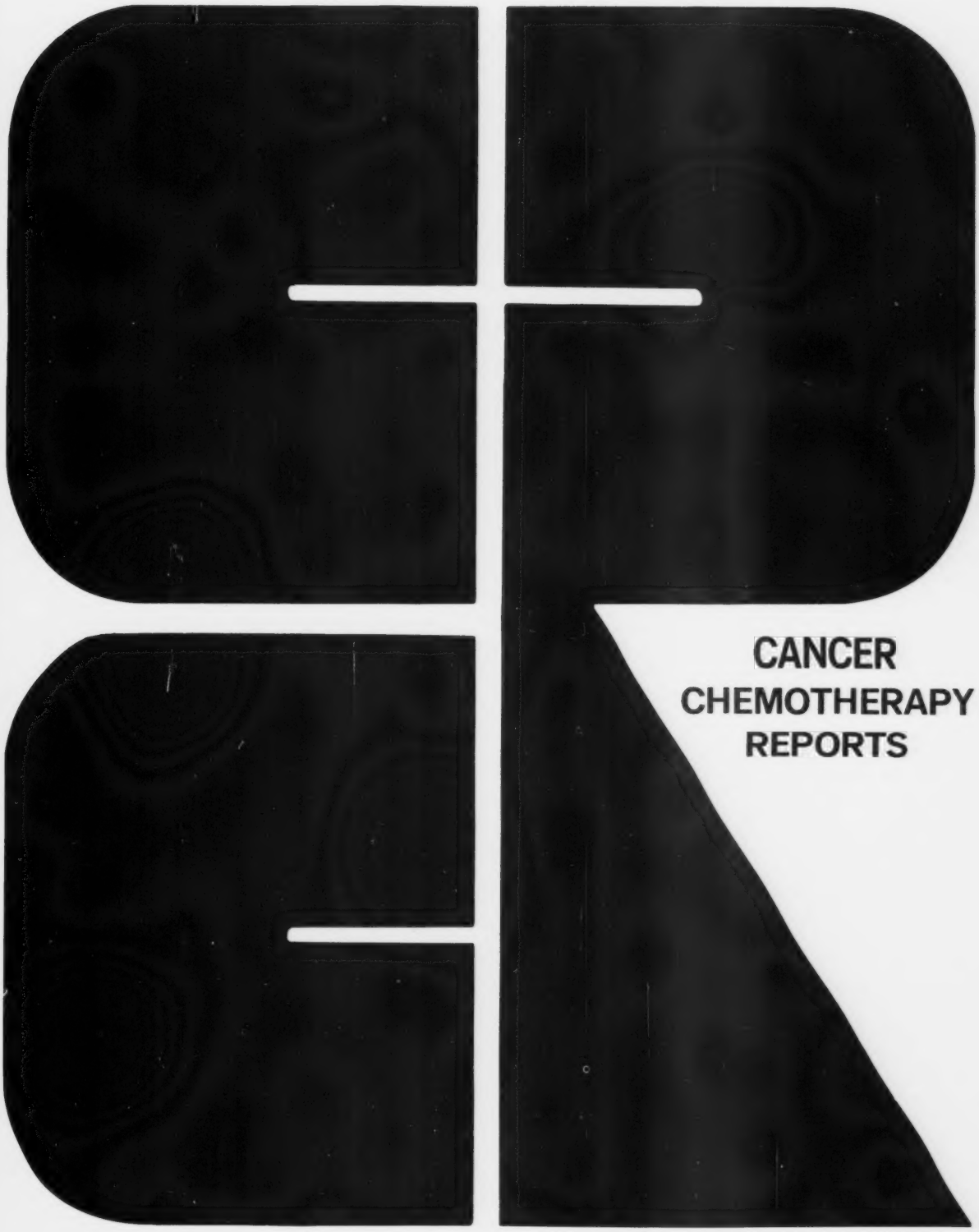

CANCER CHEMOTHERAPY REPORTS

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July/Aug 1974
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1973 Intra-Science Symposium

New Ideas in Cancer Chemotherapy

Santa Monica, California

November 28–30, 1973

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Introduction

Thomas C. Hall,[1] Symposium Organizer

The accomplishments of cancer chemotherapy were completely and elegantly presented recently at a joint conference of the American Cancer Society and the National Cancer Institute, held in New York City, and published in the journal *Cancer* for May 1973. The purpose of the present Symposium was to supplement existing overviews of accomplishments and detailed reports of specific research in progress with a thoughtful yet innovative look ahead by a small group of creative thinkers in the field. It was also to include critical reviews of certain areas in which the pace of activity might cause us to miss critical evaluations (ie, immunotherapy) and attention was to be paid to areas which might be ready for arousal from dormancy (ie, radiation sensitization). Finally, by a great leap forward of the imagination, we hoped to consider the therapeutic possibilities that might be implicit in the burgeoning knowledge of oncogenic viruses. Our hope is to find new directions based upon general principles and concepts that have recently emerged, but because they are broadly applicable, can point to the future.

[1] Oncology Section, Department of Medicine and the Advanced Therapeutics Program, Los Angeles County-University of Southern California Medical Center.

Genetic Alterations in Tumor Cells Resulting in Their Escape From the Immune Response [1]

Robert Hyman [2,3]

In the immunotherapy of tumors, one must consider both the immune response of the tumor-bearing host and the effect of the immune response on the tumor cell itself. Since the evidence indicates that the tumor-bearing animal is potentially able to respond against the tumor cell but that this response does not take place (1,2), most research is directed towards understanding how the animal responds against the tumor cell and determining the nature of the block to the immune response of the host that renders this potential response ineffective. Assuming that it will be possible to create situations where the host's immune response against the tumor is effective, then it is reasonable to ask whether the tumor cell will be able to evade the pressure of immunoselection. The problem is analogous to the problems of drug resistance occurring after chemotherapy.

Are tumor cells able to escape from immunoselection? There is much evidence to suggest that they can do so. In certain cases the escape is clearly phenotypic. The best studied of these cases with respect to a tumor antigen is the TL system (3). Cells exposed in vitro to anti-TL antibody in the absence of complement lose the ability to be killed by added antibody and complement (modulation). Loss of cytotoxicity is rapid (occurring within hours) and temperature dependent (occurring at 37 but not at 0 C). The change is reversible, since when the antibody is removed the cells regain their cytotoxic sensitivity after several days (3). A similar effect has been seen with the GCSA, an antigen induced by Gross leukemia virus (4).

It is presumed that antibody-induced modulation is the same thing as the "capping" of immunoglobulin seen on lymphocytes (5–7) and myeloma cells (8,9) in which divalent anti-immunoglobulin causes aggregation of surface immunoglobulin followed by a temperature- and energy-dependent clustering of the aggregates after which pinocytosis and degradation of the surface immunoglobulin clusters occur. The cells rapidly regain their surface immunoglobulin once the anti-immunoglobulin is removed (7).

It is also possible to obtain stable variants which, in contrast to the temporary loss of antigen characteristic of modulation, do not re-express antigen when immune selection is removed. Most of the early studies were done on "nonspecific" tumor variants which were selected to grow across histocompatibility barriers by passaging tumors in immunized animals (10,11). These variants showed a reduction in the amount of histocompatibility antigen expressed and remained stable even when grown in an immunologically neutral host (11). It was not possible to derive true negative variants which expressed no detectable H-2 antigen even after prolonged selection (12). Similar quantitative loss variants for Moloney virus tumor-specific antigens could be induced by growing a Moloney virus-induced lymphoma in preimmunized syngeneic recipients (13).

The immune mechanism by which the variants are selected in the animal (cell-mediated or humoral) is not clear. It is possible to select for quantitative variants for H-2 by in vitro treatment with antibody and complement (14). These latter variants are resistant to antibody-complement killing and have a lower quantity of H-2 antigen. The H-2 variants selected in vivo also have too few antigenic

[1] Supported by funds from the Armand Hammer Center for Cancer Biology and by grant CA-13287 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] The Salk Institute, San Diego, Calif. Scholar of the Leukemia Society.

[3] The author wishes to thank Valerie Stallings for excellent assistance with many of the experiments.

determinants for complement fixation and lysis to occur (15) and may grow in the animal due to efferent enhancement (16) in which antibody covers the antigenic sites on the cell preventing cell-mediated killing. Variants for the Moloney antigen could not be selected by antibody and complement in vitro although in vivo selection was effective (13), indicating that cell-mediated mechanisms may play a role in selecting for variants in addition to antibody and complement. If so, then it may be that a certain quantity or distribution of surface antigen is necessary for cell-mediated killing. It might be interesting to carry out repeated in vitro cell-mediated killing on a tumor line and determine the killing efficiency and amount of antigen at each cycle.

Different normal cell types are known to show qualitative and quantitative differences in their expression of cell surface antigens (17,18). It has been impossible to study the way in which the normal cell is able to regulate its expression of surface antigen, although the recent demonstration that bone marrow cells respond to thymosin by gaining TL and Thy–1 antigens (19) may provide a good model system to study this problem. The tumor cell variants which show stable changes in level of surface antigen provide convenient material to study the regulation of surface antigen expression. The approach taken by us and by others (20–22) is to derive variant cell lines which express a lower quantity of a particular surface antigen and to study the basis of the variation using the techniques of somatic genetics.

Our studies have concentrated on the normal cell surface antigen Thy–1 (formerly called θ). This antigen is expressed on thymocytes and thymus-derived lymphocytes as well as several other cell types (17,23) and is also found on thymus-derived lymphomas (23,24). There are two allelic specificities, Thy 1.1 and Thy 1.2 (formerly called θ-AKR and θ-C3H), determined by a structural gene located on chromosome 9 (linkage group II) (25,26). The chemistry of the antigen is poorly understood, although the antigenic determinant is known to occur on a membrane fragment of molecular weight 35,000 (27,28) which may be a glycolipid (28).

A number of Thy–1-bearing lymphoma lines have been established in tissue culture at The Salk Institute (29). We have derived a number of variant cell lines expressing a lowered quantity of Thy–1 by immunoselection. The general technique has been to incubate cells of a cloned Thy–1-bearing line with anti-Thy–1 and rabbit complement at 37 C, grow the survivors, and continue selection until the cells are insensitive to antibody and complement (30). The variant line is then cloned and the amount of Thy–1 present is compared to the parental line by quantitative absorption (30).

Figure 1 illustrates the sort of results obtained for a typical cell line expressing the Thy 1.1 specificity, BW5147. The variant cell line, BW5147 (Thy–), shows no detectable absorption even with as many as 5×10^7 cells, while 2.5×10^5 cells of the parental BW5147 (Thy+) line are sufficient to give 50% absorption. The variant cell has less than 0.5% of the amount of Thy 1.1 present on the parental line. Studies with other variants (30) indicate that these variants have less than 0.1% of the parental amount of Thy–1. The quantitative absorption technique used is not able to detect smaller quantities of antigen. However, since normal thymocytes have perhaps 1×10^6 molecules of Thy–1 antigen (31) and since the parental lymphomas appear roughly equivalent to thymocytes in their absorption capacity (unpublished results), it is probable that the variant cell lines have no more than 1×10^5 molecules of Thy–1 on their surface.

The surface alteration appears quite specific. With the exception of one determinant (30) there is little or no change in the expression of other surface antigens in variant cell lines. This point is illustrated for the BW5147 (Thy–) variant in table 1. Whether the two- to threefold reduction in $H\text{-}2^k$ is a real decrease or is due to an altered accessibility of the antigen to antibody is not clear. In any event, the change in the variant cell is not a nonspecific reduction in amount of or covering up of all surface antigens. Loss of Thy–1 antigen was not accompanied by changes in sensitivity to thymidine, dibutyryl cyclic AMP, hydrocortisone, thioguanine, or phytohemagglutinin (32).

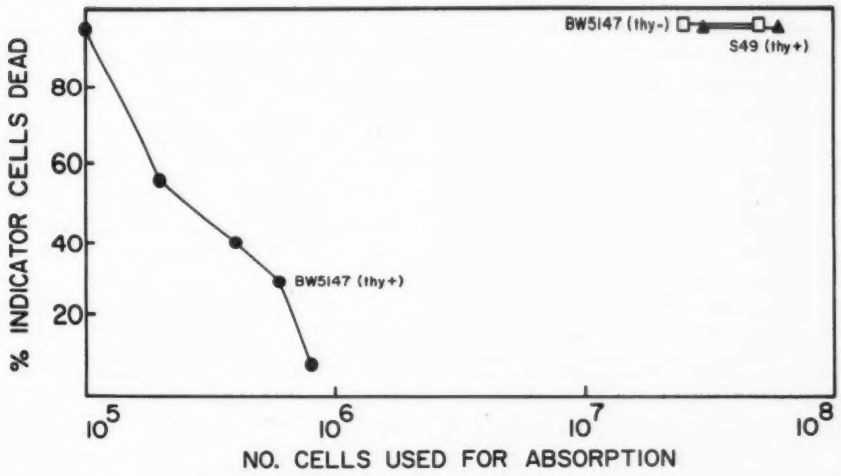

FIGURE 1.—Comparison of parental and Thy-1 variant cell lines for Thy 1.1 by quantitative absorption. Quantitative absorption was carried out as described in ref 30. BW5147 (Thy+) is the Thy 1.1-bearing parental line; BW5147 (Thy-) is a variant cell line derived by immunoselection; S49 (Thy+) is a Thy 1.2-bearing lymphoma.

TABLE 1.—Relative amount of surface antigen present on BW5147 parental and Thy-1 variant lines

Surface antigen	Cell line	Relative amount of antigen by quantitative absorption	No. of observations
Thy 1.1	BW5147 (Thy+)	1.0	2
	BW5147 (Thy-)	<0.005	
H-2^k	BW5147 (Thy+)	1.0	3
	BW5147 (Thy-)	0.3–0.5	
GCSA	BW5147 (Thy+)	1.0	2
	BW5147 (Thy-)	0.8–0.9	

A reasonable explanation for the variants is that they are pre-existing mutants in genes involved in Thy-1 synthesis which are selected for by the immunoselection techniques used. Certain data in the literature, however, make us reluctant to accept this explanation for the basis of the variants without some evidence to support it. First, the frequency of certain variants in mammalian cells, such as the loss of immunoglobulin secretion by myeloma cells in culture, is remarkably high (33). Second, certain recessive mutations which would be expected to occur much less frequently as the number of chromosome sets increases show a similar (34–36) or only somewhat decreased (37) frequency as the ploidy increases. This result has led some workers (34–36) to postulate that the variants are due to stable membrane alterations rather than to genetic changes. Third, as discussed above, certain surface antigens are known to undergo capping and endocytosis when exposed to antibody (5–9). Therefore, it is possible to conceive of "primer" models (38), in which the Thy-1 variants are induced by the selection technique itself. In such a model it is assumed that Thy-1 must be present in the membrane

as a primer for its own synthesis. Capping and endocytosis of Thy–1 during the antibody-complement selection would lead to cells which lack primer and could no longer put Thy–1 in the membrane. Such cells would be stable, phenotypic variants. If stable variants exist which have intermediate quantities of antigen, then the primer model must be modified to assume that cells with "less" primer must put less antigen on their surface, which is possible but unlikely.

The question of whether variants "pre-exist" in the population before selection has been studied in microbiology by means of indirect (or sib) selection (39) in which variants are isolated in the absence of any direct selection. This technique has been applied to the Thy–1 variants in order to determine whether antigen-loss variants pre-exist in the parental population (40). Samples of 1×10^6 cells from a number of replicate cultures of BW5147 (Thy+) were assayed for cells resistant to anti-Thy 1.1 (1:20) and rabbit complement (1:8) by determining the number of trypan blue-excluding cells after cytotoxic treatment. The culture showing the highest frequency of dye-excluding cells was chosen. The remainder of this culture (that part not used for the cytotoxic test) was then diluted so that a number of new cultures could be set up, each calculated to contain (on the average) less than one variant cell. These cultures were grown and the process was repeated. Since a variant cannot be split, those dishes which receive a variant will be enriched for variant cells, assuming that the variants are stable and that their growth rate approximates that of the parental cells (39). As can be seen from table 2, such enrichment was obtained for the Thy–1 variants (40). At cycle VII the best dish was cloned and a number of clones were screened for cytotoxicity. The clone showing least killing was then recloned and several subclones were studied by quantitative absorption. As shown in table 3, the clones isolated by indirect selection had 1%–4% of the amount of Thy 1.1 antigen present on the parental line.

TABLE 2.—Summary of indirect selection experiment (reprinted from ref 40)

Cycle	No. of dishes assayed	Calculated No. of resistants inoculated/dish	Observed No. of resistants/10^6 cells in best dish
I	24	—	9
II	9	2	73
III	10	0.2	196
IV	17	0.1	544
V	18	0.25	824
VI	15	0.03	906
VII	10	0.03	8658

TABLE 3.—Amount of Thy 1.1 antigen on subclones isolated by indirect selection (reprinted from ref 40)

Cell line *	Relative amount of Thy 1.1
BW5147 (Thy+)	1.0
BW5147 (Thy–)	<0.005
Clone 29.11	0.04
Clone 29.19	0.04
Clone 29.21	0.01

* BW5147 (Thy+) = Thy 1.1-positive parental line; BW5147 (Thy–) = Thy 1.1–negative variant isolated by direct (antibody-complement) selection against Thy 1.1; clones 29.11, 29.19, and 29.21 = subclones derived by indirect selection against Thy 1.1.

The indirect selection experiment indicates that variants which express less Thy–1 antigen than the parental line pre-exist in the population. The variants isolated by indirect selection do, however, have some detectable Thy–1 antigen in contrast to the BW5147 (Thy–) variant isolated by direct antibody-complement selection. The BW5147 (Thy–) variant, however, was isolated by repeated selection using a higher antibody and complement concentration than that used in the indirect selection experiment. Under these selection conditions variants with an intermediate level

TABLE 4.—Relative amount of Thy 1.1 antigen on clones isolated by direct immunoselection (reprinted from ref 40)

Cell line *	Relative amount of Thy 1.1 antigen
BW5147 (Thy+)	1.0
BW5147 (Thy–)	<0.002
S49 (Thy+)	<0.002
Clone 1	<0.002
Clone 2	0.006
Clone 3	0.14
Clone 4	<0.003
Clone 5	<0.002

* BW5147 (Thy+) = Thy 1.1 parental line; BW-5147 (Thy–) = Thy 1.1-negative line isolated by repeated direct immunoselection; clones 1–5 = clones isolated after subjecting BW5147 (Thy+) to 1 cycle of immunoselection using anti-Thy 1.1 (1:20) and rabbit complement (1:8) as in the indirect-selection experiment; S49 (Thy +) = Thy 1.2-bearing control line.

of Thy–1 are probably killed off. When BW-5147 (Thy+) is subjected to direct antibody complement-mediated selection using the same conditions as in the indirect-selection experiment, a spectrum of variants is obtained (table 4). These include variants with levels of Thy–1 ranging from 15% of the parental amount to less than 0.2%. The frequency of variants in this experiment was 2×10^{-5} (40).

The most reasonable interpretation of this experiment and the indirect-selection experiment is that antigenic variants which express a range of amounts of surface antigen pre-exist in the parental population. Since cytotoxic sensitivity is generally directly correlated with the amount of surface antigen present (15) (for exceptions see refs 41 and 42), a given selection condition will select for variants which express a threshold amount of antigen or less. There is no need to invoke antibody-induced loss of antigen to account for the variants although it cannot be ruled out that some variants may arise by this mechanism (40). Also, since stable variants with intermediate levels of antigen occur, these variants cannot be due to loss of primer, unless one postulates that the priming is regulated in such a way that a reduced amount of primer leads to expression of a reduced amount of surface antigen.

To study whether variants induced in different cell lines were due to the same or different defects, complementation studies were carried out by fusion of variant lines with each other (30,40). Each fusion was tested for the presence of the Thy–1 markers by quantitative absorption and for the independent H-2 marker by qualitative absorption. The four Thy–1-negative lines tested fall into three complementary classes (fig 2).

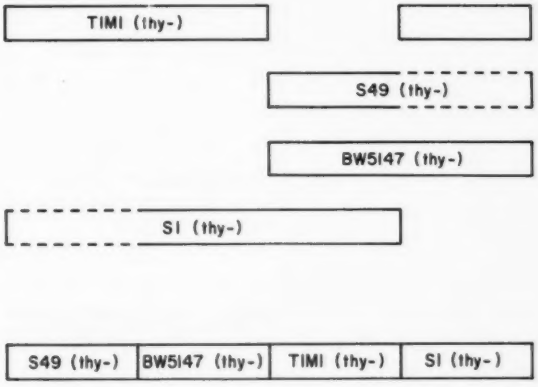

FIGURE 2.—Complementation between Thy–1-negative variants (40). Upper bars represent complementation in fusions between the cell line indicated in the bar and the cell line in the bottom bar. Spaces represent absence of complementation. The result of the fusion S1 (Thy–) × S49 (Thy–) is inferred as discussed in ref 40. [Reprinted from ref 40.]

To gain some idea as to the nature of the defects, cell fusions were done between parental and variant lines. If one fuses a parental line expressing one allele of the Thy–1 with a variant line derived from a cell line expressing the other allele, one might obtain the following results:

Result	*Interpretation*
1. Both Thy–1 alleles are absent	1. Either the defect is a dominant regulatory change in which Thy–1 synthesis is repressed in the variant *or* the variant is synthesizing a material which covers Thy–1 (or degrades it)

Result	*Interpretation*
2. Only the parental Thy-1 allele is expressed	2. The defect is a gene mutation in the Thy-1 structural gene *or* covering or degradation of only the variant Thy-1 allele is occurring
3. Both Thy-1 alleles are expressed	3. Either the variant lacks some material necessary for Thy-1 synthesis which is supplied by the parental line (probably due to structural gene changes or deletions of genes other than the Thy-1 structural gene) *or* the variant synthesizes something whose synthesis is normally repressed in the parental line which covers or degrades Thy-1

Over 30 separate fusions between different combinations of parental and variant lines (refs 30 and 40, and unpublished data) have consistently shown expression of both parental and variant Thy-1 alleles (outcome 3, above). Fusions of one variant with the same variant (ref 30 and unpublished data) never express Thy-1, indicating that the Sendai virus treatment used for fusion does not reactivate Thy-1 antigen.

The objection might be raised that, since the fusions are not assayed for surface antigens for several months after fusion, chromosomes may have been lost during this time(43). Of particular concern would be loss of a chromosome containing a possible repressor, converting outcome 1 to outcome 3. If such chromosome loss is occurring, it must be occurring invariably in all the fusions tested, which seems unlikely.

As a further test of the possibility of chromosome segregation, reselection of fusions has been performed. Chromosome losses should behave as stable deletions. Therefore, if expression of Thy-1 in fusions is due to loss of a chromosome carrying a repressor of the Thy-1 structural gene, reselection against a single Thy-1 specificity in fused cells expressing both should lead to a cell which still expresses both specificities (since the chromosome bearing the repressor has already been lost) or should lead to loss of only that specificity selected against, due to loss of the chromosome bearing the Thy-1 structural gene selected against, as occurs with selection against the H-2 antigen in heterozygous lymphomas (42,44). Such selection has been carried out on one of the fusion lines. The results (table 5) show that when one specificity is selected against, the other may also be lost, although this double loss does not invariably occur. The reselected fusions which lose both Thy-1 specificities do not behave as if they had lost a chromosome bearing a repressor before antigenic analysis had occurred. Rather, after reselection the fusions often behave as if they had lost either a gene possessing information necessary for the synthesis of both Thy-1 alleles or a repressor for a gene which synthesizes a material which either covers Thy-1 or degrades it. Presumably, those fusions losing only one allele after reselection have lost the structural gene for the respective Thy-1 allele rather than this common gene.

If the Thy-1 determinant is covered by another molecule, as appears to be the case in H-2 variants showing a similar genetic behavior (22,45), then treatment with enzymes might reveal the Thy-1 antigen. So far, treatment of Thy-1 variants with neuraminidase, hyaluronidase, glucosidases, trypsin, or chymotrypsin followed by tests for Thy-1 by absorption has not revealed Thy-1 antigen on the variant cells. Attempts to immunize against several of the Thy-1 variant lines have not given cytotoxic antisera against these variants. Although there is so far no evidence that the Thy-1 antigen is present in a masked form in the variants, such negative results do not rule out this possibility for certain, and further experiments attempting to distinguish between various alternative explanations which might give outcome 3 are in progress.

The results of the fusion experiments raise the more general question of how such recessive defects arise in diploid or heteroploid cells. There is precedent for such recessive

TABLE 5.—Expression of Thy-1 in fusions between parental and variant cells reselected for Thy-1

Fusion *	Reselected against	Clone	Expression † of—	
			Thy 1.1	Thy 1.2
F66.1	Thy 1.1	1	–	–
		2	–	–
	Thy 1.2	1	–	–
		2	–	–
		3	–	–
F78.1	Thy 1.1	1	–	–
		2	–	–
		3	+	+
F75.1	Thy 1.1	1	+	+
		2	+	+
	Thy 1.2	1	+	–
		2	+	–
		3	+	–

*F66.1 = clone of the cross of BW5147 (Thy+) × S49 (Thy–); F78.1 and F75.1 = clones of the cross of BW5147 (Thy+) × T1M1 (Thy–) (40).

†Antigens tested by qualitative absorption as described in ref 30.

defects in a number of cases in mammalian cells (for examples, *see* refs 37 and 46–48). These defects have been interpreted as mutational events (37, 47–49) and are probably due to changes in structural genes (47–49). In other cases (50,51), however, the changes leading to the recessive phenotype have been interpreted as due to changes in an "activator" rather than in a structural gene (however, *see* ref 52 for an alternate explanation of these results). In both cases it is presumed that loss of all or a portion of the relevant genome has occurred so that the cell is effectively haploid for the mutant gene (37,53). If such segregation and subsequent mutation is the explanation for the recessive mutations observed, then since multiple genes are all expressed in heteroploids (54), the similar mutation rates seen in polyploid lines (34–36) must mean that this genetic loss occurs easily so that a large number of such segregants are present in the parental heteroploid tumor lines (37). Studies of the linkage of the defects in the Thy-1 variants, comparison of the karyotype and the expression of linked markers in parental, variant and fused, and reselected cell lines, and studies of the level of Thy-1 antigen in revertant cell lines (if such revertants occur) should give more information on whether this mechanism is the one by which the surface antigen variants arise.

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Oncocognitive Autoimmunity

Thomas C. Hall [1]

The thesis I am proposing states that: (*a*) many human autoimmune diseases may result from the recognition and relatively successful elimination of foci of neoplastic cells; (*b*) the reported higher occurrence rate of neoplasms in patients with autoimmune diseases is more apparent than real, in that the emergence of frank neoplasms may occur later and more seldom in the life of such patients than would be the case had they not recognized previous tumors and suppressed them; (*c*) this concept is better suited to the current realities concerning tumor-specific transplantation antigens in man; (*d*) the autoimmune diseases often represent natural experiments in tumor immunotherapy which demonstrate the probable inadequacies of currently proposed approaches to tumor immunotherapy; (*e*) the concept of "anergy" in human cancer as currently proposed is probably incorrect, and hence therapy directed at nonspecific immune stimulation will probably not work; and finally (*f*) the alternative concepts proposed herein have great practical possibilities for early cancer detection and different, simpler types of immunotherapy in man.

Paraneoplastic Autoimmune Syndromes (1)

There are a large number of autoimmune diseases seen in conjunction with human neoplasms. These can be classified according to the tumor type with which they are associated. In general, the syndromes are commonest in epithelial tumors, which upon "invading" their basement membranes, bring epithelial material into contact with the mesenchymal immunosurveillance system. Mesenchymal tumors are less frequently associated with autoimmune syndromes. Another method of classification is by the organ attacked or damaged by the autoimmune process. There is commonly a close relationship between the site of origin of the tumor and the cells or organ damaged by the autoimmune process. Epithelial tumors are often associated with autoimmune diseases of epithelial organs, and mesenchymal tumors with connective tissue and marrow diseases. This suggests to some extent that the immunologic reactions may be in response to an antigen which is shared and is therefore probably a normal component of both the tumor and its tissue of origin. A third way of looking at the paraneoplastic syndrome-cancer relationship is in terms of the sequences involved. In many instances, the malignancy is diagnosed and the autoimmune syndrome follows. However, very often the clinical autoimmune disease precedes the clinical tumor by many months or years. This has been taken to suggest that an autoimmune disease permits the later easy emergence of a tumor, possibly by diverting the attention of the immunosurveillance system or by "exhausting" the surveillance system and thus facilitating the escape of a clone of tumor cells. In some instances these explanations may apply, but I feel that another explanation is also possible and must be considered as the data are collected. This is, that the process of tumor growth involves mutating, growing, and extending below the basement membrane and into the mesenchyme for many months or years. Such new tumor foci have been attacked, possibly effectively, by the immune system, and tumor destruction may occur. However, the immune reaction against the tumor has also spread out to damage some normal cells sharing antigenic determinants with the tumor. At this point it is well to recall that there is a great temporal delay between the neoplastic transformation of one or a few cells and the clinical diagnosis of cancer.

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For an epithelial neoplastic cell doubling once every 10 days, a log increase in cells occurs once a month, so that to reach a palpable mass containing 10^{10} cells, a minimum period of 2½ years is required. Since we know that tumor cells in general are defective, that many daughter cells die, and that such daughter cell death is greater in the unpropitious environment of a large tumor mass, the time to accumulate enough viable tumor daughter cells for a clinical diagnosis of most epithelial cells will probably be in the order of 4–8 years. It is worth recalling that small amounts of material such as radioimmunoassayable chorionic gonadotropin, immunoglobulins, alpha fetoproteins, and "carcinoembryonic antigens" have been identified as being produced by small foci of as few as 10^4 tumor cells. Hence, other products or components of tumor cells must also be released from small tumor masses.

In the case of mesenchymal tumors, the neoplastic cell has originated in the mesenchyme and shares this body compartment from the beginning with the mesenchymal immunoreactive system. There is no intraepithelial cancer-in situ stage and no necessity for "invasion" of the basement membrane. This may result in the observation that sarcomas, lymphomas, and leukemias spread widely and rapidly and with relatively few instances of autoimmune disease. Tumor cells which originate from cell types normally residing and dying in the mesenchyme should be less likely to have, as part of their normal constituents, molecules which are foreign to the mesenchyme and thus highly antigenic. On the other hand, epithelial cells are known to contain normal molecules which are forbidden to enter the mesenchymal compartment and which are perceived as antigens by the mesenchyme.

The existence, sequences, and patterns of the paraneoplastic autoimmune diseases strongly suggest that they arise because of immunologic recognition by the host of some "spatially ectopic" tissue—specific antigens carried into the mesenchyme because of the invasive character of the tumor. The high clinical incidence of neoplasms occurring after autoimmune diseases would then be seen as an artifact of the long subclinical course of most epithelial tumors. In fact, one could consider these tumor-associated autoimmune conditions to be "post-neoplastic," ie, following the recognition and suppression of the histologically normal-appearing tumor cells. Hence, a patient with an oncocognitive autoimmune disease would have fewer clinical tumors from a tissue with a constant neoplastic transformation rate than would a patient without an autoimmune disease because the latter tumors would be too undifferentiated to synthesize normal components recognizable and reactable to by the host.

Tumor-Specific Versus Tumor-Associated Antigens

A prime assumption for most of the current attempts at human immunotherapy for cancer is that there are human tumor-specific antigens, and that these can be the selectively killed specific targets of stimulated, induced, or transferred cellular or humoral immunity. There are a number of problems in this assumption, and lack of recognition of them has led to broad misstatements and erroneous attempts at therapy. Specifically, there are few, if any, instances in which rigorous tumor specificity has been demonstrated for these human tumor antigens. Table 1 shows some situations in which tumor-specific transplantation antigens (TSTAs) are thought to have been suggested. In each of these, the possibility of a tumor-associated normal product dislocated ectopically into the mesenchyme could also explain the apparent specificity. Normal epithelial cells do not broach the basement membrane and invade the mesenchyme. However, in some virus infections (eg, of the thyroid) release of normally sequestered thyroid antigens can occur, with a tissue-damaging immune thyroiditis as a result. Similarly, in trauma to the eye or testes, "spatial ectopia" or translocation of forbidden macromolecules can occur, with resultant autoimmune "sympathetic" ophthalmitis or sperm granulomas.

In the case of leukemias, the proposed tumor-specific antigens could well be constituents of the much smaller percentage of stem cells whose ontogeny is comparable to that of the leukemic cells. We know that many tumor cells express certain enzymatic (eg, thymidyl-

TABLE 1.—Proposed tumor antigens in man

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Hodgkin's disease	2 Antigens	Proto- or tropo-collagen
Sarcoma	2 Antigens	Proto- or tropo-collagen
Melanoma	Nathanson antigen	Tyrosine oxidase, melanin
Carcinoma, cervix	Epithelial antigen	Keratohyalin
Brain	Tumor-specific antigen	Brain protein

ate synthetase) or synthetic (eg, muramidase) capacities which are more characteristic of the tissues of the growing embryo or of the few blastogenic "stem" or repair cells than of the normal differentiated cells of the adult tissues. Hence, analyzing the leukemic cells en masse, one might mistakenly assume that a leukemic-specific antigen had been formed, when in reality it was a tissue-specific, ontogeny-phase-specific, temporally ectopic macromolecule which would also always be found in the very small number of normal early blast cells in the marrow.

There are a number of tumor-associated ectopic macromolecules found in other conditions which are not usually thought of as "tumor-antigens" but which could well have been called such had they been stumbled upon in the quest for TSTAs. Some of these are shown in table 2. Dr. Marvin Romsdahl of the M. D. Anderson Hospital found circulating tyrosine oxidase enzyme activity in the plasma of melanoma patients (2). If he had used an immunologic approach such as that employed by Dr. Larry Nathanson's team in Boston (3) he could have described the same molecule as a "tumor-specific" antigen. We still do not know whether Nathanson's and Romsdahl's materials are the same ectopic macromolecules examined by different techniques. Calcitonin could well have been confused with a tumor-specific antigen from medullary thyroid cancer had it been looked for as a unique antigen rather than as a normal hormone. Much confusion has occurred because it was assumed that macromolecules found by immunologic methods were new TSTAs. Part of this confusion was due to lack of recognition that tumor cells commonly share many characteristics of their normal tissue-of-origin counterpart cells: it is this *similarity* which permits the pathologist to classify most tumors on the basis of shared characteristics with normal tissues. Part of the confusion rose out of a lack of realization that human cancer should be part of the same biologic universe as animal cancer and that tumor-*specific* antigens required a rigorous delineation. In addition, part of the confusion rose out of the failure to appreciate that a macromolecule could be an antigen in a

foreign host and yet not antigenic in the cancer patient. There are (table 3) surely a large number of tumor-associated macromolecules circulating in patients which do not cause autoimmune disease yet which would be antigenic to foreign hosts. These could easily be picked up as "tumor-specific" by tests using heterogenic antisera.

TABLE 2.—Heterotopic macromolecules in search of "antigenicity"

Tumor	Macromolecules
Carcinoma, lung	Fetal membrane protein Solubilized keratohyalin
Adrenal	Fetal adrenal protein
Leukemia	f-Hemoglobin
Carcinoma, breast	α-Lactalbumin β-Lactoglobulin Casein
Carcinoma, stomach	Gastric mucoprotein
Carcinoma, ovary	Mucoprotein
Carcinoma, colon	Meconium
Sarcoma, bone	Hydroxyproline polypeptides
Brain	Cerebroside-protein complexes

TABLE 3.—Known heterotopic macromolecules which might have been mistaken for tumor antigens

Tumor	Macromolecules
Carcinoma, lung	Physiologically inactive parathyroid hormone Adrenocorticotropic hormone
Carcinoma, kidney	Anaerythropoieitin Erythropoieitin
Myeloma	M-globulin
Prostate	Acid phosphatase
Leukemia	Muramidase B_{12}-binding protein
Carcinoma, medullary thyroid	Thyrocalcitonin

Differences Between Human and Animal Cancer Antigens

Carcinogenesis in rodents has been related to virus-induction of neoplasia (including the effect of radiation upon thymic viruses) and to chemicals (fig 1). In the former instance, the oncogenic virus produces an antigen which is membrane-associated and virus-specific. This results in all tumors induced by one virus sharing a single antigen, even though they may be quite different histologically, as in the case of those induced by polyoma virus (4). To my knowledge, this has not yet been shown unequivocally for human cancer. In a given cancer patient, we would expect one antigen for her breast cancer and another for her leukemia, even though we postulate that her breast cancer and her leukemia are related.

Most animal RNA virus-induced tumors are mesenchymal and share T-antigens. However, not all T-antigen-labeled cells are neoplastic. In man, sarcoma viruses, sarcoma antigens, neuroblastoma antigens, and neuroblastoma-related immune changes have been detected in the relatives of children with this tumor. Definitive tests have not been done to date with appropriate normal control tissues to justify the assumption that these antigens should be considered anything but spatially antigenically ectopic normal macromolecules.

Chemically induced animal cancers have a specific antigen for each new tumor nodule (5). Thus, one guinea pig can have a dozen

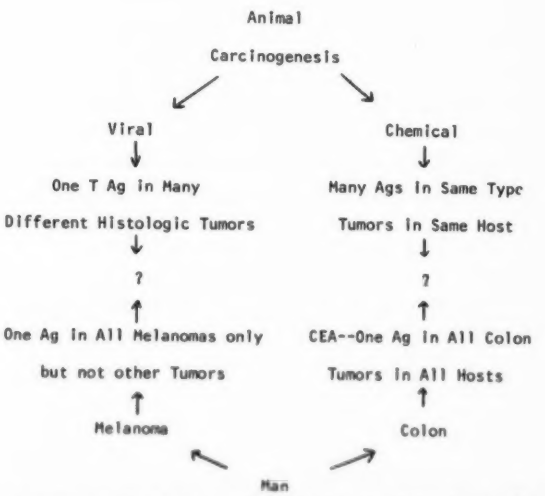

FIGURE 1.—Defects in relating human tumor immunity to animal models.

antigenically different sarcomas after one carcinogen exposure. These tumor antigens are also quite distinct for every tumor found in every different animal. This appears to be generally true of all species in which chemicals have induced tumors. Extrapolated to man, it would mean that tumors thought to be carcinogen induced, such as those occurring in the exposed epithelia and increasing in frequency with the age of the patient and the duration of exposure to the environment, would each contain a totally different antigenic makeup. This is not what has been described; instead we have had one colon cancer antigen (CEA) described for *all* colon cancers in *all* patients, suggestions of similar single antigens for bladder, kidney, cervix, lung, and breast cancers, and others for melanoma and Hodgkin's disease.

It can be seen that the type of tumor antigen usually described for man is a third type, primarily related to the histologic type of cancer rather than to the carcinogen. It can also be seen that the type of cancer antigenicity described for animals is quite different from that which has been described for man to date, and that no real demonstration of animal type viral or chemical tumor antigens has been adduced for man. Three conclusions arise from this: first, that the antigens described for man are *not* apt to be TSTAs but rather tissue-specific, tumor-associated, heterotopic oncomolecules which are temporally or spatially ectopic; second, that there will, in time, be discovered true carcinogen-related neo-antigens, since man is subject to the same biology and even more environmental carcinogens than laboratory animals; third, that the inability to describe such TSTAs to date, and the fact that most carcinogen-induced human tumors are apt to occur in the covering epithelia from whence only the least differentiated will escape into the mesenchyme, both suggest that the carcinogen-induced TSTAs will be found to be excessively weak and probably non-exploitable immunotherapeutically.

The histology of the RNA tumor viruses in animals involves vertical transmission, mesenchymal tumors, and T-antigens present on both tumor and normal cells. These suggest that tumors of childhood primarily may be found to be RNA-virus induced, since tumors of young children arise before sufficient time has elapsed for chemicals to work. They are mostly leukemias and sarcomas of mesenchymal origin, and the Hellströms have described antigens in older relatives without cancer, suggesting vertical transmission of an infectious factor. However, all of these factors militate against developing a very effective immunotherapy of such postulated pediatric, tumor-specific, viral-transplantation antigens. This is because vertical transmission suggests the presence of antigens in the embryonic state with resulting antigen-specific immune paralysis. Also, sharing of the T-antigen by normal cells would probably result in an autoimmune destruction of normal cells as a necessary price for any antitumor immunotherapy based upon exploitation of the T-antigen. Finally, in animals, viral neoplastic transformation can take place in cells which lack T-antigens, so there could well be transformed human leukemic or sarcoma cells which would escape the most effective cytotoxic immunotherapy based upon the viral TSTA.

Is the Tumor Patient "Anergic"?

Whether or not there are TSTAs, and especially if all tumor antigens are weak, it might be well to stimulate the host's immune system if it is not already reacting properly to the presence of tumors and their antigens. Many investigators report that tumor patients show "anergy" and that this is proportionate to the stage of the disease (6). Other reports suggest that one of the primary effects of chemical carcinogens is depression of the function of the immunocompetent cells of the exposed host (7). As a result, nonspecific stimulation of the immune system has been suggested as a logical therapy. However, much of the data cited in support of the concept of generalized energy is based upon the patient's lack of reaction to new biologic and chemical antigens such as dinitrochlorobenzene (DNCB) and key-hole limpet hemocyanin. It is conceivable that the intact reactivity of the immunocytes is masked by the presence of large amounts of circulating tumor-associated

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Brain	Tumor-specific antigen	Brain protein

ate synthetase) or synthetic (eg, muramidase) capacities which are more characteristic of the tissues of the growing embryo or of the few blastogenic "stem" or repair cells than of the normal differentiated cells of the adult tissues. Hence, analyzing the leukemic cells en masse, one might mistakenly assume that a leukemic-specific antigen had been formed, when in reality it was a tissue-specific, ontogeny-phase-specific, temporally ectopic macromolecule which would also always be found in the very small number of normal early blast cells in the marrow.

There are a number of tumor-associated ectopic macromolecules found in other conditions which are not usually thought of as "tumor-antigens" but which could well have been called such had they been stumbled upon in the quest for TSTAs. Some of these are shown in table 2. Dr. Marvin Romsdahl of the M. D. Anderson Hospital found circulating tyrosine oxidase enzyme activity in the plasma of melanoma patients (2). If he had used an immunologic approach such as that employed by Dr. Larry Nathanson's team in Boston (3) he could have described the same molecule as a "tumor-specific" antigen. We still do not know whether Nathanson's and Romsdahl's materials are the same ectopic macromolecules examined by different techniques. Calcitonin could well have been confused with a tumor-specific antigen from medullary thyroid cancer had it been looked for as a unique antigen rather than as a normal hormone. Much confusion has occurred because it was assumed that macromolecules found by immunologic methods were new TSTAs. Part of this confusion was due to lack of recognition that tumor cells commonly share many characteristics of their normal tissue-of-origin counterpart cells: it is this *similarity* which permits the pathologist to classify most tumors on the basis of shared characteristics with normal tissues. Part of the confusion rose out of a lack of realization that human cancer should be part of the same biologic universe as animal cancer and that tumor-*specific* antigens required a rigorous delineation. In addition, part of the confusion rose out of the failure to appreciate that a macromolecule could be an antigen in a

foreign host and yet not antigenic in the cancer patient. There are (table 3) surely a large number of tumor-associated macromolecules circulating in patients which do not cause autoimmune disease yet which would be antigenic to foreign hosts. These could easily be picked up as "tumor-specific" by tests using heterogenic antisera.

TABLE 2.—Heterotopic macromolecules in search of "antigenicity"

Tumor	Macromolecules
Carcinoma, lung	Fetal membrane protein Solubilized keratohyalin
Adrenal	Fetal adrenal protein
Leukemia	f-Hemoglobin
Carcinoma, breast	α-Lactalbumin β-Lactoglobulin Casein
Carcinoma, stomach	Gastric mucoprotein
Carcinoma, ovary	Mucoprotein
Carcinoma, colon	Meconium
Sarcoma, bone	Hydroxyproline polypeptides
Brain	Cerebroside-protein complexes

TABLE 3.—Known heterotopic macromolecules which might have been mistaken for tumor antigens

Tumor	Macromolecules
Carcinoma, lung	Physiologically inactive parathyroid hormone Adrenocorticotropic hormone
Carcinoma, kidney	Anaerythropoieitin Erythropoieitin
Myeloma	M-globulin
Prostate	Acid phosphatase
Leukemia	Muramidase B_{12}-binding protein
Carcinoma, medullary thyroid	Thyrocalcitonin

Differences Between Human and Animal Cancer Antigens

Carcinogenesis in rodents has been related to virus-induction of neoplasia (including the effect of radiation upon thymic viruses) and to chemicals (fig 1). In the former instance, the oncogenic virus produces an antigen which is membrane-associated and virus-specific. This results in all tumors induced by one virus sharing a single antigen, even though they may be quite different histologically, as in the case of those induced by polyoma virus (4). To my knowledge, this has not yet been shown unequivocally for human cancer. In a given cancer patient, we would expect one antigen for her breast cancer and another for her leukemia, even though we postulate that her breast cancer and her leukemia are related.

Most animal RNA virus-induced tumors are mesenchymal and share T-antigens. However, not all T-antigen-labeled cells are neoplastic. In man, sarcoma viruses, sarcoma antigens, neuroblastoma antigens, and neuroblastoma-related immune changes have been detected in the relatives of children with this tumor. Definitive tests have not been done to date with appropriate normal control tissues to justify the assumption that these antigens should be considered anything but spatially antigenically ectopic normal macromolecules.

Chemically induced animal cancers have a specific antigen for each new tumor nodule (5). Thus, one guinea pig can have a dozen

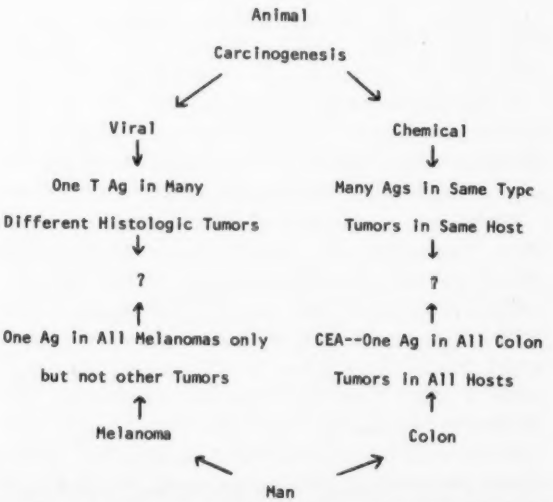

FIGURE 1.—Defects in relating human tumor immunity to animal models.

antigenically different sarcomas after one carcinogen exposure. These tumor antigens are also quite distinct for every tumor found in every different animal. This appears to be generally true of all species in which chemicals have induced tumors. Extrapolated to man, it would mean that tumors thought to be carcinogen induced, such as those occurring in the exposed epithelia and increasing in frequency with the age of the patient and the duration of exposure to the environment, would each contain a totally different antigenic makeup. This is not what has been described; instead we have had one colon cancer antigen (CEA) described for *all* colon cancers in *all* patients, suggestions of similar single antigens for bladder, kidney, cervix, lung, and breast cancers, and others for melanoma and Hodgkin's disease.

It can be seen that the type of tumor antigen usually described for man is a third type, primarily related to the histologic type of cancer rather than to the carcinogen. It can also be seen that the type of cancer antigenicity described for animals is quite different from that which has been described for man to date, and that no real demonstration of animal type viral or chemical tumor antigens has been adduced for man. Three conclusions arise from this: first, that the antigens described for man are *not* apt to be TSTAs but rather tissue-specific, tumor-associated, heterotopic oncomolecules which are temporally or spatially ectopic; second, that there will, in time, be discovered true carcinogen-related neo-antigens, since man is subject to the same biology and even more environmental carcinogens than laboratory animals; third, that the inability to describe such TSTAs to date, and the fact that most carcinogen-induced human tumors are apt to occur in the covering epithelia from whence only the least differentiated will escape into the mesenchyme, both suggest that the carcinogen-induced TSTAs will be found to be excessively weak and probably non-exploitable immunotherapeutically.

The histology of the RNA tumor viruses in animals involves vertical transmission, mesenchymal tumors, and T-antigens present on both tumor and normal cells. These suggest that tumors of childhood primarily may be found to be RNA-virus induced, since tumors of young children arise before sufficient time has elapsed for chemicals to work. They are mostly leukemias and sarcomas of mesenchymal origin, and the Hellströms have described antigens in older relatives without cancer, suggesting vertical transmission of an infectious factor. However, all of these factors militate against developing a very effective immunotherapy of such postulated pediatric, tumor-specific, viral-transplantation antigens. This is because vertical transmission suggests the presence of antigens in the embryonic state with resulting antigen-specific immune paralysis. Also, sharing of the T-antigen by normal cells would probably result in an autoimmune destruction of normal cells as a necessary price for any antitumor immunotherapy based upon exploitation of the T-antigen. Finally, in animals, viral neoplastic transformation can take place in cells which lack T-antigens, so there could well be transformed human leukemic or sarcoma cells which would escape the most effective cytotoxic immunotherapy based upon the viral TSTA.

Is the Tumor Patient "Anergic"?

Whether or not there are TSTAs, and especially if all tumor antigens are weak, it might be well to stimulate the host's immune system if it is not already reacting properly to the presence of tumors and their antigens. Many investigators report that tumor patients show "anergy" and that this is proportionate to the stage of the disease (6). Other reports suggest that one of the primary effects of chemical carcinogens is depression of the function of the immunocompetent cells of the exposed host (7). As a result, nonspecific stimulation of the immune system has been suggested as a logical therapy. However, much of the data cited in support of the concept of generalized energy is based upon the patient's lack of reaction to new biologic and chemical antigens such as dinitrochlorobenzene (DNCB) and key-hole limpet hemocyanin. It is conceivable that the intact reactivity of the immunocytes is masked by the presence of large amounts of circulating tumor-associated

antigen, and experiments involving repeated lymphocyte washings have demonstrated that they may then be shown to be immunocompetent. It is also possible that in the presence of great excesses of tumor-associated antigens each new group of immunocompetent cells would react first to the tumor antigens and thus become immunologically "committed" cells unable to react to new antigens applied as skin-test substances. Removal of tumor would result in a fall in tumor-associated antigen levels and would free the next crop of immunocompetent cells to respond to the skin test neoantigens. Hence "anergy" may be too encompassing a term to describe the phenomenon which consists of normal reactivity to tumor-associated antigens, with resulting relative hyporeactivity to nontumor neoantigens. Such a view is more compatible with the fact that other studies have reported that tumor patients show positive skin tests to intradermal injections of soluble extracts of membranes from their own leukemic cells (8), breast cancers (9), or colon cancers (10). The possibility that autologous intracellular cytoplasmic proteins would provide even more suitable antigens has yet to be extensively studied. Such tissue-specific, tumor-associated antigens should be most suitable, based on the comments made above concerning the tissue-associated nature of the human so-called tumor antigens prepared to date. Furthermore, this concept has been strengthened by reports that patients with breast cancer respond positively to extracts of normal breast tissue. The reported lack of responsiveness of leukemic patients to antigens from normal white blood cells is not surprising: the proper normal tissue counterpart to the leukemic blast cell would be the few normal stem cells in the marrow, a population naturally impossible to isolate in pure form, so that there is little reason to think the untransfused leukemic patient should react to normal white blood cells.

If, then, the tumor patient is not truly "anergic," there would seem to be little success to be gained by attempts at nonspecific immunofactors, as for example, with Bacillus Calmette-Guérin (BCG). In this vein, it is important to recognize that success with BCG vaccination in animals has been restricted to those animal systems in which there was (*a*) a clearly specific chemically induced antigen, and (*b*) direct contact between BCG and the tumor cells, so that tumor cell death and macrophage processing were possible. In man, the only clearcut BCG responses have been in similar situations, ie, when melanomas were directly injected with BCG, and possibly in acute leukemia, in which large numbers of leukemic cells in the small skin vessels were brought into contact with BCG in the midst of a scarified coagulum. There is, to my knowledge, no evidence of a beneficial effect of BCG operating at a distance from the tumor cell and through "nonspecific" stimulation of the immune system (11).

These data suggest that it might be reasonable to alter the host's immune reactivity to the tumor by direct alteration of the antigenicity of the tumor cell. Dr. M. B. Sahasrabudhe (personal communication) has described such an alteration of leukemic blast cells by incubating them with dinitrofluorobenzene, following which some host immune destruction of the leukemic cells has been described.

Ulcerative Colitis as an Example of Oncocognitive Autoimmunity

There is now an overwhelming body of data demonstrating that CEA is not a tumor-specific antigen but represents a family of glycocalyceal glycoproteins from various epithelial cells. A small amount is normally released into the body constantly and results in the observed "normal" levels of up to 2.5 ng/ml of plasma. Large amounts are found whenever epithelial products are retained in the body instead of being discharged via a duct lumen into the outside environment. Hence, meconium is full of CEA, as are the aspirated contents of breast cysts and as is normal gastrointestinal mucus when protected from fecal bacterial digestion, as in the collected secretions of the ileal segments used in ileal bladders. Hence, CEA is neither carcinoma-specific nor embryonic. Nor is it usually antigenic, since there have been no confirmatory reports of circulating antibodies and only one report of possibly autoimmune glomerulonephritis in which CEA might be incriminated as the antigen (12).

As summarized by Watson (13), the lymphocytes of patients, especially children, with ulcerative colitis have been reported to have circulating noncytotoxic antibodies to epithelial antigenic components of normal colons; since these are not cytotoxic, they probably do not function either in immune surveillance or in the pathogenesis of ulcerative colitis. However, cytotoxic lymphocytes also circulate in ulcerative colitis, are specific for normal colonic epithelium, and can be blocked by a large excess of antigen from *Escherichia coli* 0119: B14 (13).

Lymphocyte reactions to colon-specific antigenic material should be greater when the colon epithelium is most differentiated, and Sion and Friedell (14) have reported that there is more lymphocyte infiltration around polyps than around colon cancers. The Hellströms (15) reported that lymphocytes from patients with either progressive or cured colon cancer are cytotoxic for other immature colon cells, both normal and malignant, an effect which can be blocked by addition of serum from patients with colon cancer. The latter suggests immune paralysis by excess antigen. All of these findings are compatible with the hypothesis that small foci of malignant, immature, but recognizably colonic epithelial cells excite the production of an immune lymphocytic response as they broach the basement membrane and enter the mesenchyme. In most instances, these recently transformed cancer cells are properly killed, but in a few instances their similarity to the crypt cells is so great that autoimmune destruction by anticancer lymphocytes of some normal epithelium occurs. In some patients with a cancer diathesis, repeated malignant clones occur, especially in the lower colon where the cumulative effects of exogenous carcinogens would be greatest. Thus ensues an ongoing autoimmune process which we call ulcerative colitis, maximal also at the lower rectal end of the colon. In time, the continued production of mutational clones leads to some which are just undifferentiated enough to escape the surveillance system, and frank cancer occurs. This may be hastened by the general immunosuppressive effects of the carcinogen and the immune deficiency secondary to the protein depletion occasioned by the ulcerative colitis. Thus, the ulcerative colitis is seen as a postneoplastic oncocognitive autoimmune process. It may be that the comparable surveillance of small bowel neoplasms is of greater antitumor effectiveness. This is suggested by the fact that lymphocytes from patients with Crohn's disease are also cytotoxic against normal colon epithelium, and such patients have humoral antibodies as well (16). In Crohn's disease migratory inhibitory factor production was induced in all patients by the CEA in normal meconium, whereas this reaction was seen only occasionally in patients with ulcerative colitis (17). Such more explicit reactivity in the upper gastrointestinal tract and in Crohn's disease may be related to the lower incidence of cancer in the small bowel and in patients with Crohn's disease as compared with the higher incidence of cancer in patients with ulcerative colitis.

There are a number of unexamined questions with respect to these concepts:

1. There is a suggestion that both the severity of ulcerative colitis and the incidence of colon cancer are higher in the younger age groups.
2. The high incidence of colon cancer seems to be independent of the state of activity of the ulcerative colitis.
3. I have found no studies in which the lymphocytes from patients with ulcerative colitis or Crohn's disease have been studied for their cytotoxicity for colon cancer epithelia.

One might expect that Crohn's disease lymphocytes would react very much more effectively than those of ulcerative colitis, and that the colon cancer cells from a patient with both ulcerative colitis and colon cancer would tend to escape cytotoxicity in vitro as they had in vivo.

Clinical Uses of Oncocognitive Autoimmunity

Other tumor-associated, tissue-specific, and possibly oncocognitive autoimmune diseases include myasthenia, pure red cell aplasia, and dermatomyositis. Patients with these diseases

may carry large numbers of killer lymphocytes against many clones of malignant colonic epithelial cells. They might be able to react more effectively against the malignant colonic epithelial cells of other patients with colon cancer, and a search for such reactivity should be made. If found, it might permit the transfusion of lymphocytes from an ulcerative colitis or Crohn's disease donor to be used to treat other patients with colon cancer.

Similarly, such colon-reactive lymphocytes might be found in patients with small tumors and thus incorporated into a specific early-detection system for colon cancer. Furthermore, if, as suspected, the antigen involved is tissue-specific to normal colon and becomes antigenic only when the colon tumor cells enter the mesenchyme, it might be possible to immunize a foreign species such as the horse using normal human colonic material. The cytotoxic lymphocytes resulting could be transfused into the colon cancer patient and result in tumor destruction, the foreign cells ultimately being rejected by a host-versus-graft reaction. Similarly, normal epithelial constituents from breast, lung, kidney, etc, may contain antigens which are able to induce lymphocytes cytotoxic to corresponding cancers if infused into the cancer patient. Antibodies induced in foreign hosts might be more cytotoxic than those of the original host, and if not, they might be tagged with radioisotopes both for tumor localization and cell-killing. Since they, and the cytotoxic lymphocytes, would react primarily with tumor cells which had invaded the mesenchyme, one should expect relatively little damage to normal epithelia protected by its basement membrane.

The concept of oncocognitive autoimmunity might permit identification of those noncancer patients who had successfully rejected tumors. Lymphocytes from patients with dermatomyositis could be reacted with antigens from normal lung, stomach, and other tissues, and thus indicate whether the dermatomyositis patient was harboring cancer in one of these organs. Such dermatomyositis-reactive lymphocytes might also be used to treat other patients with frank neoplasms. Other autoimmune diseases may indicate a group of patients with lymphocytes programmed to have successfully rejected a number of cancers and thus of potential benefit to patients with clinical cancer.

The concept of oncocognitive autoimmunity is closely tied to the concept of TSTAs and might permit early diagnosis of a number of malignancies by permitting the radioimmunoassay detection, in large populations, of small amounts of circulating products which were either epithelial in origin and spatially ectopic in the bloodstream or were the products of cells blocked in ontogeny at a level of synthesis usually repressed in the adult, ie, temporally as well as spatially ectopic. One of the antigens that could be sought in the attempt to examine this theory might be an antigen from fetal adrenal cortex. Since this organ involutes after birth and is replaced by the adult adrenal, it seems reasonable to expect that the cells of the adrenal cortical cancer might synthesize and release some antigens shared with the fetal adrenal. Similarly, fetal pulmonary hyaline (or its antibodies) might be found in the tumors or plasma of lung cancer patients.

In summary, it is proposed that:

1. Many autoimmune diseases result from the recognition and destruction of malignant cells, in the course of which similar normal tissues are damaged (fig 2) instead of tumor-specific antigens.
2. The common antigen between normal and malignant tissues in such autoimmune syndromes is most apt to be a normal epithelial product which is in the wrong place or is being synthesized at the wrong time in the host's lifespan.
3. The autoimmune diseases may represent a necessary but unavoidable side effect of effective immunotherapy. Immunotherapy which does not cause autoimmunity may not be effective.
4. To date, all tumor antigens have appeared to be tissue-specific and hence less susceptible to therapeutic exploitation than in the case of animal tumors with specific viral or chemically induced tumor antigens.

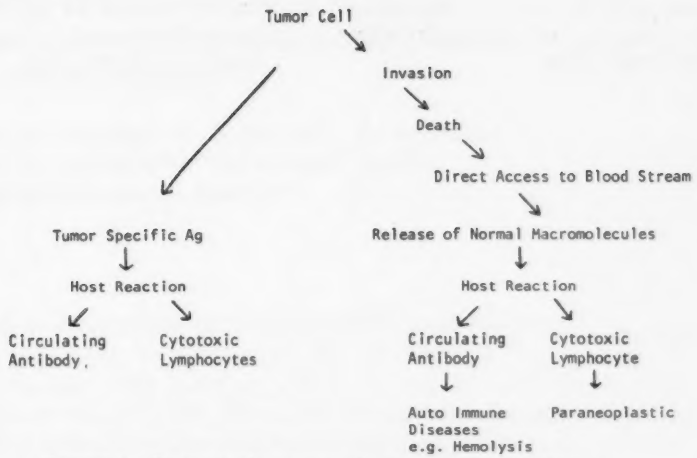

FIGURE 2.—Heterotopic macromolecules vs tumor antigens.

5. So-called anergy in cancer patients may not exist.
6. In the absence of TSTAs and generalized anergy, current attempts at tumor-specific immunotherapy and nonspecific stimulation of immunity are not apt to be effective.
7. There are potential uses of the concepts of oncocognitive autoimmunity and the heterotopic oncomolecule in cancer detection and therapy.
8. A number of specific experiments are suggested to test these hypotheses.

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Synthesis and Biologic Examination of Some Derivatives of 2,2′-Anhydro-1-(β-D-arabinofuranosyl)cytosine Hydrochloride (NSC-145668) [1,2]

J. G. Moffatt,[3] E. K. Hamamura,[3] A. F. Russell,[3] M. Prystasz,[3] J. P. H. Verheyden,[3] K. Yamaguchi,[4] N. Uchida,[4] K. Sato,[4] S. Nishiyama,[4] O. Shiratori,[4] and K. Katagiri [4]

SUMMARY

A brief survey of the reactions of 2-acyloxyisobutyryl halides with nucleosides is presented. In particular, the reactions of 2-acyloxyisobutyryl chloride with cytidine and a variety of cytidine analogs and derivatives lead to the formation of an homologous series of 2,2′-anhydro-1-(3′-*O*-acyl-β-D-arabinofuranosyl)cytosine hydrochlorides (*33*). Acylation of 2,2′-anhydro-1-(β-D-arabinofuranosyl) cytosine hydrochloride (*26*, cyclocytidine) forms the corresponding 3′,5′-diacyl-cyclocytidines (*35*) in high yield. Methods are developed for the hydrolysis of these acylated cyclonucleosides (*33* and *35*) to the corresponding 3′-acyl- or 3′,5′-diacyl- derivatives of cytosine arabinoside (*34* and *36*). These various series of compounds have been shown to possess in vitro antiviral activities as well as in vivo activities against L1210 leukemia and Ehrlich ascites carcinoma. The antileukemic activities of certain derivatives are very high relative to the parent nucleosides and a preliminary investigation of different modes of administration is reported.

[Cancer Chemother Rep 58:451–469, 1974]

In 1964, while investigating a series of pyrrolizidine alkaloids, A. R. Mattocks came upon an unusual acylation reaction (1). In following up this observation Mattocks showed that simple 1,2-diols such as ethylene glycol *(1)* react with 2-acetoxy-2-methylbutyryl chloride *(2)* to form 2-chloroethyl acetate *(3)* and 2-hydroxy-2-methylbutyric acid (*4*) in high yield (1). A similar abnormal reaction occurred between *2* and propane-1,3-diol to form 3-chloropropyl acetate. Mattocks has demonstrated that the formation of chloroacetates from diols is a characteristic reaction of 2-acetoxyacyl chlorides bearing two alkyl substituents on the α-carbon. This is presumably due to severe steric hindrance towards nucleophilic attack at the acyl chloride carbonyl group and a tentative reaction mechanism has been proposed (1).

1 2 3 4

Our own interest in nucleoside chemistry, and, in particular, our studies relating to the synthesis of nucleosides containing halogenated

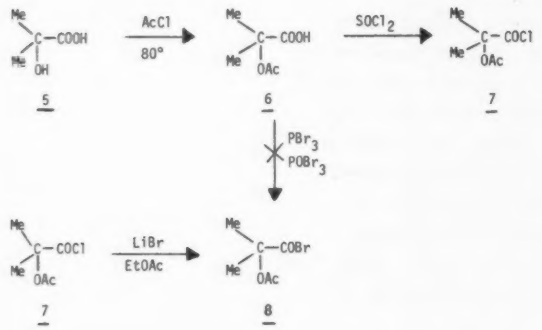
[1] Contribution No. 125 from the Institute of Molecular Biology, Syntex Research, Palo Alto, Calif.

[2] NSC-145668: CAS reg. No. 10212-25-6; 6*H*-furo-[2,3′:4,5]oxazolo-[3,2-*a*]pyrimidine-2-methanol, 2,3,3*a*,-9*a*-tetrahydro-3-hydroxy-6-imino-, monohydrochloride; *O*³,2′-cyclocytidine, monohydrochloride; cyclocytidine.

[3] Institute of Molecular Biology, Syntex Research, Palo Alto, Calif.

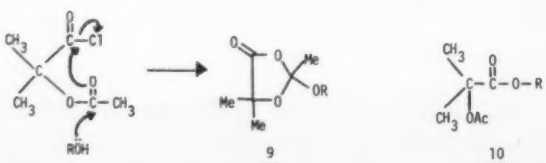
[4] Shionogi Research Laboratories, Osaka, Japan.

sugars (2,3), led us to investigate this reaction further. Since the reagent *2* contains a chiral center we have preferred to use 2-acetoxyisobutyryl chloride (*7*) in our work. The latter reagent is readily prepared on a large scale from commercially available 2-hydroxyisobutyric acid (*5*) via sequential treatment with acetyl chloride and thionyl chloride (4). As will be seen later, in some of our studies we require 2-acetoxyisobutyryl bromide (*8*) rather than the corresponding acyl chloride (*7*). We have found that *8* is more readily prepared by reaction of *7* with lithium bromide in ethyl acetate than by bromination of the intermediate hydroxy acid (*6*) with phosphorus tribromide or phosphorus oxybromide (4).



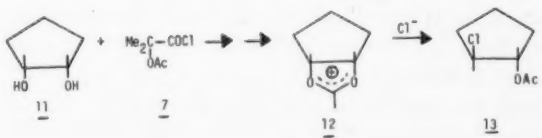
5 6 7 7 8

Before progressing to a systematic study of the reactions of *7* with diols such as nucleosides, we examined some isolated primary and secondary alcohols. In general, simple alcohols such as *p*-nitrobenzyl alcohol or cyclohexanol react rapidly with *7* at room temperature to give 2,5,5-trimethyl-1,3-dioxolan-4-one-2-yl ethers such as *9*. These rather unusual products, which are labile to both mild acid and base, clearly arise, as suggested by Mattocks (1), via initial nucleophilic attack of the alcohol upon the acetoxycarbonyl group rather than the acyl chloride. Under certain circumstances of solvent, etc, the isomeric 2-acetoxyisobutyrate esters (*10*) can also be formed (4).



9 10

In order to clarify the stereochemical requirements of the reaction, *7* was reacted under identical conditions with the pure *cis* and *trans* isomers of cyclopentane-1,2-diol and cyclohexane-1,2-diol (4). The results of these experiments were striking and indicate a clear steric course for the reaction. Thus, *cis*-cyclopentane-1,2-diol (*11*) reacted rapidly with *7* at room temperature to give exclusively *trans*-2-chlorocyclopentyl acetate (*13*) in 95% yield. On the other hand, *trans*-cyclopentane-1,2-diol gave a complex mixture of products including only traces of *13*. These products included a variety of dioxolanone ethers and acetate esters and their distribution was markedly solvent dependent. A very similar pattern emerged from the reactions of *7* with *cis*- and *trans*-cyclohexane-1,2-diol, the *cis* isomer being once again converted almost quantitatively to *trans*-2-chlorocyclohexyl acetate while the *trans*-diol gave a complex mixture of products. From the above results it is clear that the formation of chloroacetates is the unique reaction of *7* with *cis*-diols and proceeds with inversion of configuration at one center. Based upon these observations, and others from our work with nucleosides, we have proposed that the essential feature of this reaction is the conversion of the *cis*-diol (*11*) into an acetoxonium ion (*12*) which is then opened by chloride ion giving the *trans*-chloroacetate (*13*). A mechanistic rationale for the formation of the acetoxonium ion (*12*) has been considered in detail elsewhere (4).

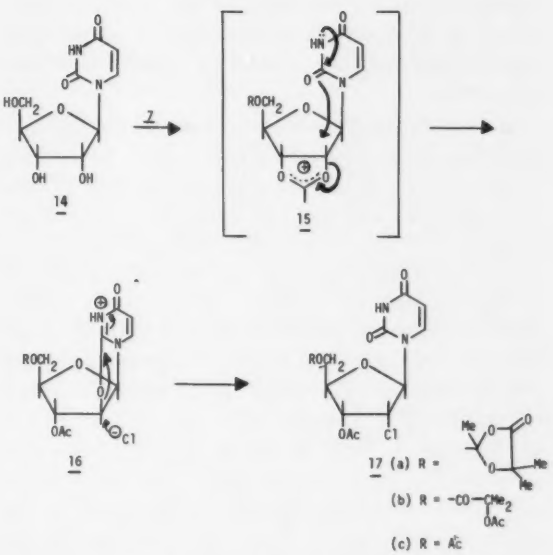


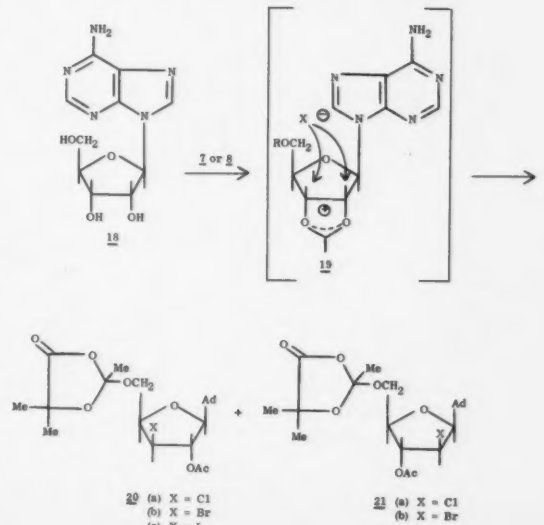
11 7 12 13

With the realization that a *cis*-diol is the required structural feature for reaction with 2-acetoxyisobutyryl chloride, we turned to a general examination of various nucleosides. It was first shown that uridine (*14*) reacts readily with *7* in a variety of inert solvents, complete reaction usually requiring 30–60 minutes at roughly 80 ° (4). The predominant product in solvents such as acetonitrile and dimethylformamide proved to be the 3′-*O*-acetyl-2′-chloro-

2′-deoxyuridine derivative (*17a*), the 5′-oxygen being substituted as a trimethyldioxolanone ether. Comparable reactions in nitromethane, or in the absence of solvent, gave the identical 3′-*O*-acetyl-2′-chloro-2′-deoxyuridine in which the 5′-hydroxyl group was present as its 2-acetoxyisobutyryl ester (*17b*). Yet another reaction in glacial acetic acid gave the corresponding 5′-acetate (*17c*) in high yield. In each of the above products the position and stereochemistry of the chloro and acetoxy functions could be confirmed by nuclear magnetic resonance (NMR) spectroscopy and by conversion upon brief treatment with sodium methoxide to crystalline 2′-chloro-2′-deoxyuridine which has been unambiguously synthesized by independent means (3,5). It will be noted that unlike the model experiments with *cis*-cycloalkane-1,2-diols, the reactions with uridine led to chloroacetates with overall retention of stereochemistry. This can be explained by assuming that the intermediate 2′,3′-acetoxonium ion (*15*) is first opened by attack of the 2-carbonyl group of the uracil ring giving the protonated 2,2′-anhydro-1-(3′-*O*-acetyl-β-D-arabinofuranosyl)uracil derivative (*16*). Subsequent attack by chloride ion at the 2′-position of *16* then gives the observed 2′-chloro-2′-deoxynucleoside (*17*) with overall retention of configuration. The intermediacy of the acetoxonium ion (*15*) was confirmed by direct reduction of this species with sodium borohydride to 2′,3′-*O*-ethylideneuridine, and the crystalline cyclonucleosides (*16*, R = H and R = trimethyldioxolanone) could be isolated from brief reactions in nitromethane and dimethylformamide respectively (4).

It is well known that N^3 of the purine ring in adenosine can participate in reactions at C_5' of the sugar forming N^3,5′-cyclonucleosides (6). On the other hand, there are only a few suggestions that N^3 can participate in reactions at C_2' or C_3' of the sugar moiety of purine nucleosides (7,8). Accordingly, one might expect that the reaction of purine nucleosides such as adenosine (*18*) with *7* would lead to an intermediate 2′,3′-acetoxonium ion (*19*) which would be normally opened to give *trans*-chloroacetates. In fact, the reaction of adenosine (*18*) with *7* required roughly 30–60 minutes at 80 ° or 18 hours at 37 °. In addition to a small amount of adenine resulting from glycosidic cleavage of the nucleoside, two isomeric products were formed in a ratio of 9:1. These proved to be the 2′-*O*-acetyl-3′-chloro-3′-deoxy-β-D-xylofuranosyl nucleoside (*20a*) and its 3′-*O*-acetyl-2′-chloro-2′-deoxy-β-D-arabinofuranosyl isomer (*21a*) resulting from direct opening of the intermediate acetoxonium ion (*19*) by chloride ion (9). In both cases the 5′-hydroxyl



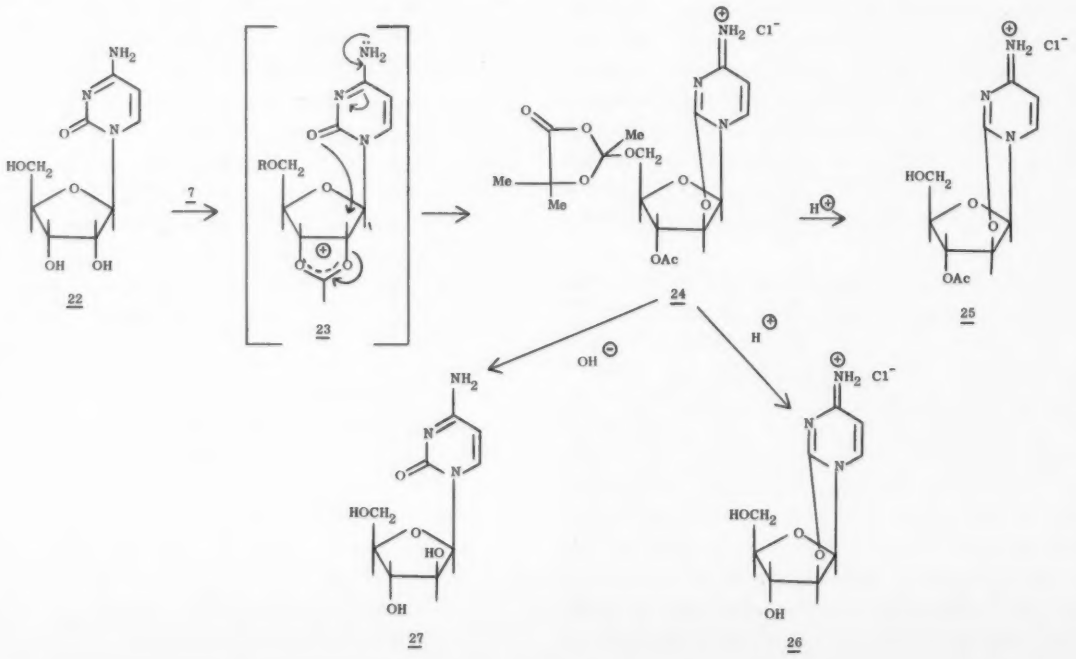


group was present as a dioxolanone ether and the preferential formation of the 3′-chloro compound is presumably a consequence of relative steric hindrance at $C_{3'}$ and $C_{2'}$ due to the adenine ring.

A comparable reaction between adenosine and 2-acetoxyisobutyryl bromide (*8*) proceeded readily at room temperature giving the bromo acetates (***20b*** and ***21b***) once again in a ratio of roughly 9:1. The iodo acetate (***20c***) could also be prepared from adenosine and *7* in the presence of an excess of sodium iodide (10). Very similar types of products were obtained from the reaction of *7* and *8* with a variety of other purine nucleosides such as tubercidin (11), formycin (11), inosine (12), and N^2, 5′-dibenzoylguanosine (12). In each case the 3′-halo-D-xylo isomer (***20***) predominated over its 2′-halo-D-arabino- counterpart (***21***). In the case of the reaction of formycin (11), the 5′-oxygen in the products was exclusively substituted as the 2-acetoxyisobutyryl ester rather than as the trimethyldioxolanone ether. Compounds of the general types ***20*** or ***21*** have proved to be versatile intermediates for the synthesis of 2′-deoxy-, 3′-deoxy-, and 2′,3′-dideoxy-nucleosides (9,11, 12), as well as of 2′,3′-anhydronucleosides (9, 11,12) and of nucleosides containing 2′,3′- and 3′,4′-unsaturated sugars (10).

The reaction of cytidine (***22***) with *7* in acetonitrile at 80 ° took an interesting course.[5] The reaction mixture was initially heterogeneous but became homogeneous after 5–10 minutes. Almost immediately thereafter a crystalline product began to separate. After a total of 30 minutes the remaining material was precipitated with ether and the total solid was crystallized from methanol-acetone. By a combination of analytic techniques, in particular ultraviolet and NMR spectroscopy, the product was readily identified as 2,2′-anhydro-1-(3′-*O*-acetyl-β-D-arabinofuranosyl)cytosine hydrochloride (***25***). While the yield of ***25*** was reasonable (60%-70%) from small-scale reactions, attempted scale-up led to some difficulties. This problem was circumvented by conducting the reaction at room temperature, a clear reaction mixture being obtained in about 3 hours without separation of crystalline product. Addition of ether then precipitated the 5′-dioxolanone de-

[5] Russell AF, Prystasz M, Hamamura EK, et al. Manuscript submitted to the *Journal of Organic Chemistry*.



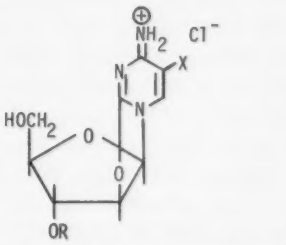
rivative (*24*) in essentially quantitative yield. Removal of the labile dioxolanone group was readily effected by brief treatment with 0.05 M methanolic hydrogen chloride. Subsequent crystallization then gave *25* identical to that from the 80 ° reaction above in an overall yield of 90% from cytidine and with a purity in excess of 95%. It is presumed that *24* is also an intermediate in the reaction at 80 ° but that the labile dioxolanone group is lost during workup and crystallization.

As in the case of the reactions of uridine, the formation of the 2,2′-anhydronucleosides (*24* and *25*) is a consequence of intramolecular opening of an initially formed 2′,3′-acetoxonium ion intermediate (*23*). In the cytidine series, the 2,2′-anhydronucleosides are very stable in acidic solution and are not readily opened by nucleophilic attack to form 2′-chloro-sugars as was the case with the corresponding uridine derivatives.

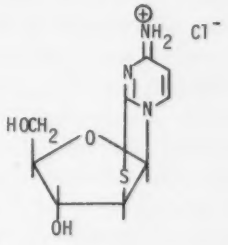
Crude *24* is also a convenient intermediate for the preparation of other compounds of current interest. Thus, treatment with methanolic hydrogen chloride under somewhat more vigorous conditions than were used for conversion to *25* led to the formation of 2,2′-anhydro-1-(β-D-arabinofuranosyl) cytosine hydrochloride (*26*, cyclocytidine), a compound that has become the subject of extensive examination as an antitumor agent of low toxicity (13–15). This method has been applied to the preparation of *26* in overall yields of 70%-80% from cytidine on a kilogram scale and would appear to be the synthesis of choice for this compound. Alternatively, treatment of crude *24* with dilute bases such as ammonium hydroxide leads to cleavage of the anhydro linkage and the acetyl group giving the well-known antileukemic agent cytosine arabinoside [6] (*27*) (16–18) in an overall yield of 73% from cytidine. The comparable hydrolysis of unsubstituted *26*, which has been prepared by a variety of methods, to *27* has been described previously (19–22).

Since the acetylated anhydronucleoside showed significant activity against DNA viruses and L1210 leukemia, we investigated the preparation of a variety of base analogs. Accordingly, 5-fluorocytidine, 5-chlorocytidine, 5-bromocytidine, 5-iodocytidine, 5-dimethylaminocytidine, 5-methylcytidine, 2-thiocytidine, 6-azacytidine, and N^4,N^4-dimethylcytidine were all reacted with *7* in acetonitrile (*see* footnote 5). In each case the corresponding 2,2′-anhydro-1-(3′-*O*-acetyl-β-D-arabinofuranosyl) cytosine hydrochloride derivative or analog was formed in satisfactory yield. In some cases the products crystallized directly from the reaction medium but it was usually necessary to first remove the 5′-dioxolanone ether by brief treatment with methanolic hydrogen chloride. This procedure sometimes led to partial cleavage of the 3′-*O*-acetyl group and in those cases, in order to most readily obtain crystalline products, the acidic treatment was prolonged so as to complete the deacetylation. By these general procedures the crystalline derivatives *28–31* were prepared and characterized.

It should be pointed out that the 5-bromo- (*28c*) and 5-iodo- (*28d*) derivatives have recently been prepared by an alternative route



28 (a) X = F, R = Ac
(b) X = Cl, R = H
(c) X = Br, R = H
(d) X = I, R = H
(e) X = Me, R = H
(f) X = NMe_2, R = Ac



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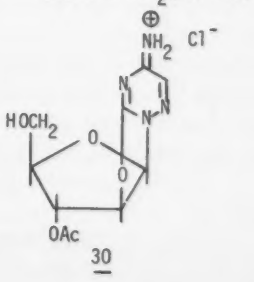

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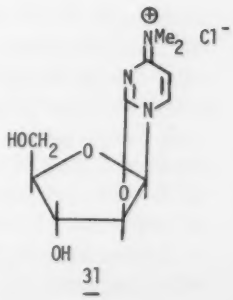


31

[6] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

(23). In addition, a synthesis of the deacetylated analog of ***28a*** has been described and this compound has been shown to have activity against L1210 leukemia in mice (24). Compounds ***28–31*** have also been evaluated by us for both antiviral and antileukemic properties. Without elaboration it is sufficient to say that while the 5-haloderivatives ***28a***, ***28c***, and ***28d*** showed reasonable activity against DNA viruses (50% plaque reduction of vaccinia virus at micromolar concentrations of 0.45, 1.50, and 1.35), the others showed little activity at the concentrations tested. Similarly, ***28a***, ***28d***, and ***29*** showed modest activities against L1210 leukemia in mice, but less than that of cyclocytidine (***26***) itself.

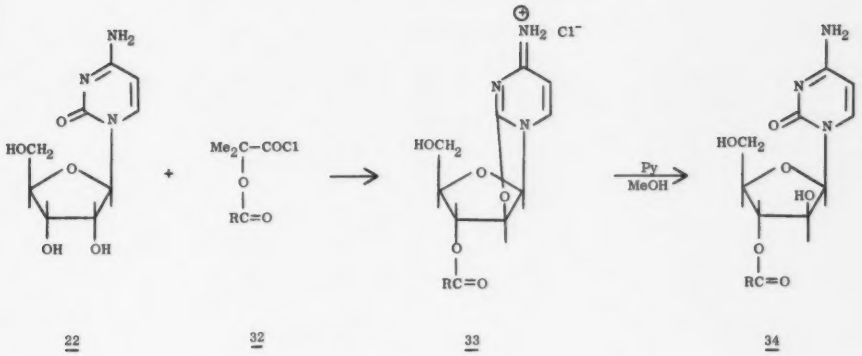
Since ***26*** is an ionic substance and is known to be unstable as the free base (20), cellular permeability might well prove to be somewhat of a problem with compounds of this sort. Accordingly, we initiated efforts to prepare more lypophilic derivatives in this series. One such series of compounds was prepared by a direct extension of the method used above for the 3′-*O*-acetyl derivative ***25***. Thus, an homologous series of 2-acyloxyisobutyryl chlorides (***32***) was prepared by the same route used for ***7*** except that various aliphatic or aromatic acyl chlorides were used in place of acetyl chloride. Many members of this series (***32***) could be readily purified by vacuum distillation. Frequently, however, the higher homologs were prepared using equimolar amounts of ***5*** and the appropriate acyl chloride followed by treatment with excess thionyl chloride and were used directly without purification. In these ways a series of reagents (***32***) was prepared in which the acyl group varied in size from acetyl to behenoyl (ie, C_2–C_{22}) and in general considering only acids with even numbers of carbon atoms. Each of these derivatives was reacted with cytidine in acetonitrile at 25–80 °. In each case the appropriate 2,2′-anhydro-1-(3′-*O*-acyl-β-D-arabinofuranosyl)cytosine hydrochloride (***33***, 3′-acyl-cyclocytidine) was formed and isolated in crystalline form, generally in yields of 50%–70% and usually without the need for chromatographic purification.

A second homologous series of derivatives was prepared by direct acylation of ***26*** with the same series of acyl chlorides used for preparation of ***32***. Since ***26*** is rather insoluble in most organic solvents and is also unstable in the presence of bases even as weak as pyridine, these acylation reactions were done using the acyl chloride in dimethylacetamide without added base. In many cases the resulting 2,-2′-anhydro-1-(3′,5′-di-*O*-acyl-β-D-arabinofuranosyl) cytosine hydrochloride (***34***, 3′,5′-diacyl-cyclocytidine) crystallized directly from the reaction mixture in high yield. If this was not the case, then crude ***34*** could be precipitated by addition of ether and then crystallized.

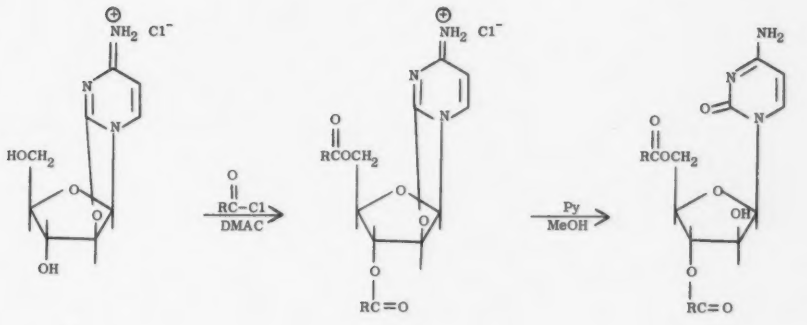
Finally, we were concerned with developing a method by which the 2,2′-anhydro-nucleoside linkage in both the 3′- and 3′,5′-diesterified series (***33*** and ***35***) could be cleaved without loss of the acyl groups. In the case of the more soluble, lower acyl derivatives this could be effected by treatment with sodium bicarbonate-sodium carbonate in aqueous dioxane. For the less-soluble derivatives, however, it was found that treatment with pyridine-methanol (1:1) at 60 ° provided a convenient route. Since *N*-methylpyridinium chloride was formed during this procedure we presume that the reaction involves initial methanolysis followed by demethylation by attack of pyridine.

For the moment, the precise nature of the first step in the above sequence is not certain and the intermediate might be either ***37a*** or ***37b***. Using this procedure the various 3′-esters and 3′,5′-diesters of cyclocytidine (***33*** and ***35***) have been converted into the corresponding esters of cytosine arabinoside (***34*** and ***36***, 3′-acyl- and 3′,5′-diacyl-cytosine arabinoside) in satisfactory yields.

Previous work has shown that it is possible to prepare a variety of 5′-esters of cytosine arabinoside by direct acylation (25). Some of the derivatives so obtained, particularly the 5′-*O*-palmitate (17) and the 5′-adamantoate (26), have been shown to possess enhanced immunosuppressive and antileukemic properties relative to the parent substance. In addition, it has been shown that 1-(2,3,5-tri-*O*-butyryl-β-D-arabinofuranosyl)cytosine shows significant activity against L1210 leukemia when administered on a multiple-treatment schedule (27). The synthesis of a few 2′- and 3′-*O*-monoesters of

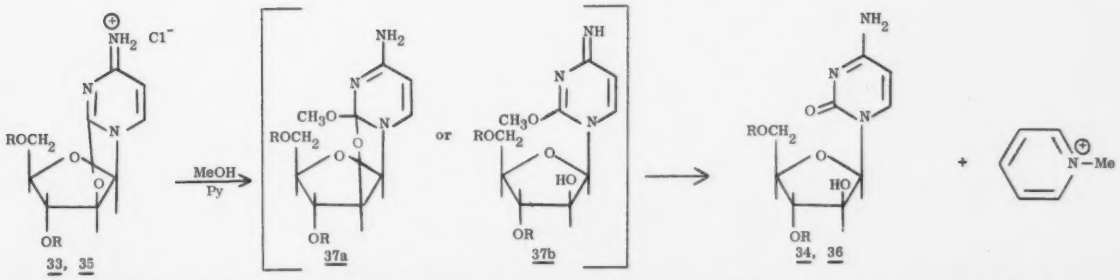


22 32 33 34



26 35 36

In 32–36, R = CH_3 to $C_{21}H_{43}$

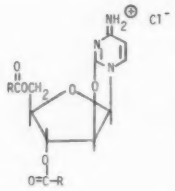


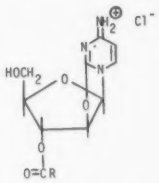
33, 35 37a 37b 34, 36

cytosine arabinoside has also been described via partial acylation of a suitably N^4,5′-blocked intermediate (28). A difficult chromatographic separation of the 2′- and 3′-esters was, however, necessary and the yields of purified products were only 2%–5%. The synthetic methods presently reported make the 3′-esters (*33* and *35*) and the 3′,5′-diesters (*34* and *36*) readily available compounds for biologic study.

As an initial in vitro assay we have examined the antiviral activities of the various esters (*33–36*). These activities were initially studied using a disc diffusion assay with BGM African green monkey kidney cells (30) infected with both DNA and RNA viruses. The conclusion reached was that, like cytosine arabinoside itself (31), compounds of types *33–36* show marked activity against DNA but not RNA viruses. In order to put these studies on a more quantitative basis, the various compounds were then examined by a plaque-reduction assay using both the HF strain of herpes simplex and the IHD strain of vaccinia virus. The results of these studies are shown in tables

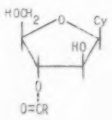
TABLE 1.—Antiviral activity in tissue culture

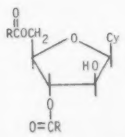




3'-Substituted cyclocytidine	μM Concentrations resulting in 50% plaque reduction of— Vaccinia	Herpes simplex	3',5'-Disubstituted cyclocytidine	μM Concentrations resulting in 50% plaque reduction of— Vaccinia	Herpes simplex
—	0.41	0.70	—	0.41	0.70
3'-*O*-Acetyl	0.73	2.25	3',5'-Di-*O*-acetyl	0.69	2.40
3'-*O*-Butyryl	0.59	2.05	3',5'-Di-*O*-butyryl	1.55	4.90
3'-*O*-Hexanoyl	0.23	1.55	3',5'-Di-*O*-hexanoyl	—	—
3'-*O*-Octanoyl	0.19	0.72	3',5'-Di-*O*-octanoyl	0.56	1.35
3'-*O*-Decanoyl	0.15	1.05	3',5'-Di-*O*-decanoyl	0.32	1.10
3'-*O*-Lauroyl	0.15	0.64	3',5'-Di-*O*-lauroyl	0.70	3.45
3'-*O*-Myristoyl	0.34	1.35	3',5'-Di-*O*-myristoyl	1.75	6.05
3'-*O*-Palmitoyl	1.20	5.50	3',5'-Di-*O*-palmitoyl	28	>30
3'-*O*-Stearoyl	1.60	5.44	3',5'-Di-*O*-stearoyl	>30	>30
3'-*O*-Arachidyl	>30	>30	3',5'-Di-*O*-arachidyl	>30	>30
3'-*O*-Behenoyl	18.5	>30	3',5'-Di-*O*-behenoyl	>30	>30

TABLE 2.—Antiviral activity in tissue culture





3'-Substituted cytosine arabinoside	μM Concentrations resulting in 50% plaque reduction of— Vaccinia	Herpes simplex	3',5'-Disubstituted cytosine arabinoside	μM Concentrations resulting in 50% plaque reduction of— Vaccinia	Herpes simplex
—	0.07	0.16	—	0.07	0.16
3'-*O*-Acetyl	0.58	1.45	3',5'-Di-*O*-acetyl	—	—
3'-*O*-Butyryl	1.20	2.80	3',5'-Di-*O*-butyryl	1.15	5.20
3'-*O*-Hexanoyl	0.48	1.50	3',5'-Di-*O*-hexanoyl	—	—
3'-*O*-Octanoyl	0.11	0.64	3',5'-Di-*O*-octanoyl	0.22	2.60
3'-*O*-Decanoyl	0.05	0.28	3',5'-Di-*O*-decanoyl	0.35	0.78
3'-*O*-Lauroyl	0.05	0.25	3',5'-Di-*O*-lauroyl	2.05	7.20
3'-*O*-Myristoyl	0.18	0.64	3',5'-Di-*O*-myristoyl	5.70	>10
3'-*O*-Palmitoyl	0.14	0.67	3',5'-Di-*O*-palmitoyl	30.0	>30
3'-*O*-Stearoyl	0.38	0.70	3',5'-Di-*O*-stearoyl	>30	>30
3'-*O*-Arachidyl	>30	>30	3',5'-Di-*O*-arachidyl	>30	>30
3'-*O*-Behenoyl	>30	>30	3',5'-Di-*O*-behenoyl	>30	>30

1 and 2. From both tables it is clear that vaccinia virus is somewhat more sensitive to acylated derivatives of both the cyclocytidine (*33* and *35*) and cytosine arabinoside (*34* and *36*) types than is herpes simplex virus. With all four types of compounds, the introduction of short chain acyl groups results in somewhat diminished activity relative to the parent nucleosides. As the acyl groups increase in size, however, the antiviral activities are enhanced, reaching optimum values in the vicinity of the decanoyl esters. Further increases in the chain length of the acyl groups lead to decreased activities, particularly in the cases of the diester derivatives (*35* and *36*). The greatly reduced activities of the higher acyl derivatives are quite probably a reflection of the very low solubilities of these derivatives in the aqueous tissue culture medium. Further studies on the antiviral activities of compounds of these sorts in both in vitro and in vivo situations will be reported at a later date.

Our major interest at this time was, however, a demonstration of the antitumor activities of these four series of compounds. For this purpose we have principally examined two systems, namely BDF_1 mice infected intraperitoneally (ip) with 1–2 × 10^5 L1210 leukemia or Ehrlich ascites carcinoma cells. For routine purposes the various nucleosides were administered ip as solutions or suspensions in an aqueous medium containing 0.9% sodium chloride, 0.4% polysorbate 80, 0.5% low-viscosity sodium carboxymethylcellulose, and 0.9% benzyl alcohol. Treatment was generally started 24 hours after infection. For L1210-infected mice the effectiveness of the treatment was determined from the average percent increase in lifespan (%ILS) of the treated group (eight to ten animals) relative to that of an infected, but non-drug-treated, control group. Under these conditions the average life expectancy of the control group was close to 8 days and the experiments were terminated after 30 days. Since with many of the compounds tested a number of the treated mice were still alive after 30 days, the %ILS reported in these cases is a minimum figure. In the following figures, points corresponding to situations where mice were still alive after 30 days are indicated with an arrow (↑), and the number of living mice per group is recorded.

Since the minimum effective dose for the various compounds varies widely, it is not particularly instructive to tabulate %ILS figures for a series of compounds at a single dose level. We have, however, determined dose-response profiles for many compounds, and generalities can be drawn from selected examples.

Figures 1–4 show dose-response curves for compounds of types *33*, *34*, *35*, and *36* with acyl groups containing eight, 14, 18, and 22 carbon atoms. In each case the compound in question is administered as a single ip injection 24 hours after infection with L1210 cells as above. As positive controls, the responses to the parent unsubstituted nucleosides, cyclocytidine (*26*) and cytosine arabinoside (*27*), are also recorded. From these figures it can be seen that under these conditions cyclocytidine and cytosine arabinoside show modest maximal activities only at doses of 2000 mg/kg, and that above this level the response falls off, presumably due to drug toxicity. The lower acyl derivatives of these compounds (eg, the octanoyl derivatives, *33–36*, R = C_7H_{15}) show very low activities, whereas the longer chain esters show remarkably different profiles. In most cases maximum activities were achieved with greater than 250% ILS and generally with a predominance of 30-day survivors. The dosage ranges over which maximal activity was achieved, however, varied widely. Thus, comparable high activity was observed with the 3′-C_{14}, C_{18}, and C_{22} esters of cyclocytidine (fig 1) at single doses of 1000 mg/kg, but there were marked differences in the responses at higher doses due to the onset of drug toxicity. In the case of the 3′,5′-diesters of cyclocytidine (fig 2), maximal activity of the C_{14} ester was displaced to much lower doses (200 mg/kg) but toxic effects were apparent at 500 mg/kg. On the other hand, 3′,5′-distearoylcyclocytidine (*35*, R = $C_{17}H_{35}$) was maximally effective only at higher doses but showed no sign of toxicity at even 4000 mg/kg. In general, the 3′-acyl-cytosine arabinoside derivatives (fig 3) showed high activity at 100–200 mg/kg but toxicity followed at higher doses. 3′-Behenoyl-cytosine arabinoside (*34*, R = $C_{21}H_{43}$)

was, however, somewhat of an exception since it showed a very broad profile with little toxicity. In a rather similar way (fig 4), 3′,5′-dimyristoyl-cytosine arabinoside (*36*, R = $C_{13}H_{27}$) was active at much lower doses than was its C_{18} analog, although the latter showed no observable toxicity at high doses.

It is well known that cytosine arabinoside is rapidly inactivated in vivo by enzymatic deamination thus necessitating complex multiple dosage schedules for optimal effectiveness (31). This drawback can be partially alleviated by the use of cyclocytidine, which is itself inert to the action of cytidine deaminase (32). An alternate approach to increasing the biologic half-life of cytosine arabinoside has been through the use of lipophilic 5′-esters which are resistant towards deamination and appear to serve as depot forms of the parent nucleoside (17,27). We have examined different modes of administration for a number of our compounds. Figures 5–7 show the results of a comparison of the antileukemic effects of three derivatives of cyclocytidine (3′-myristate, 3′,5′-dimyristate, and 3′,5′-distearate), each administered in a single ip dose on Day 1, or in consecutive doses on Days 1, 2, 3, 4, and 5 by the ip, subcutaneous (sc), and oral routes. In the

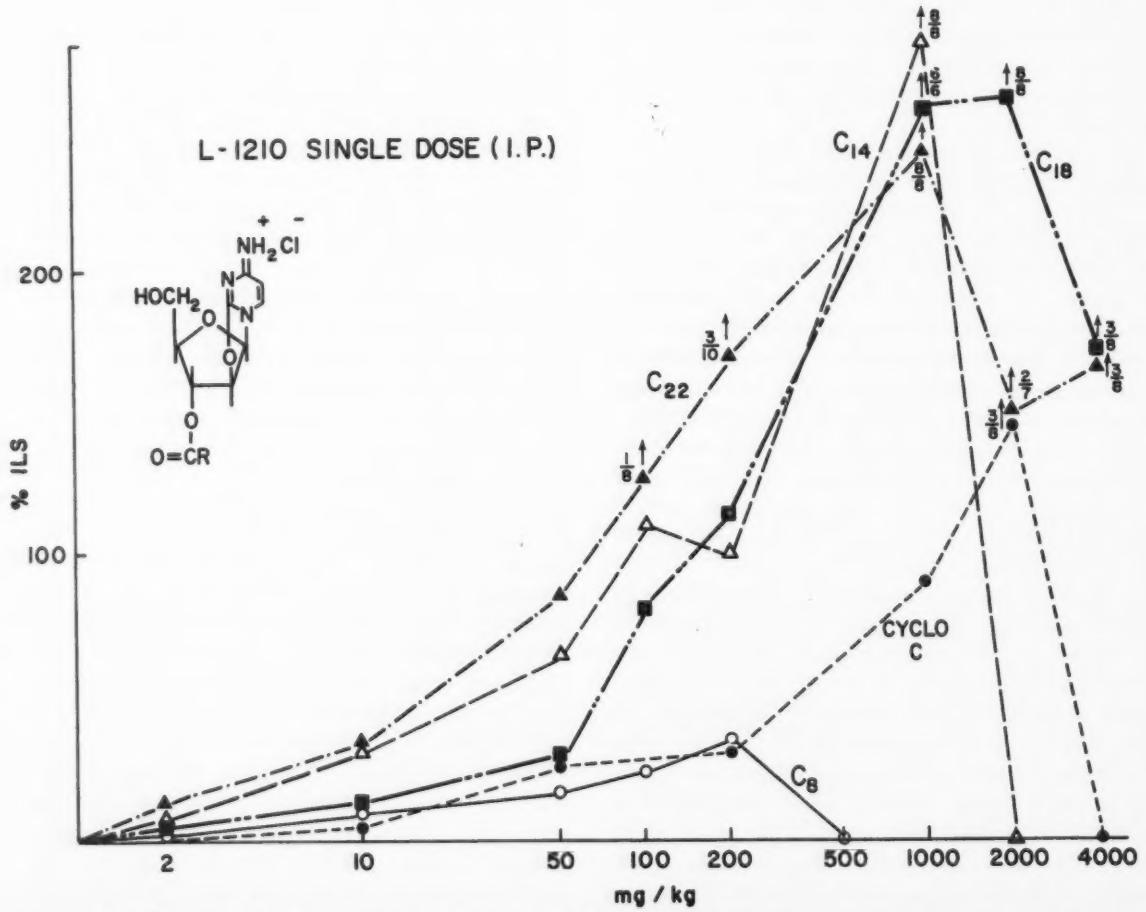


FIGURE 1.—Effects of single ip injections of 3′-*O*-acyl-cyclocytidine hydrochlorides on mice bearing L1210 leukemia. The number of carbon atoms in the acyl group is indicated for each curve. *See* text for additional information.

case of the divided regimen the indicated dose is the sum of the five daily doses so that the effects can be directly compared with those of a single equivalent treatment on Day 1. It can be seen from figures 5–7 that the divided ip dose is significantly more effective than the single equivalent administration. Once again, the profiles shown by the different compounds are quite distinct from one another. The efficacy of divided sc doses is also quite variable. Thus, the effect of sc administration of 3′-myristoyl-cyclocytidine (fig 5) is essentially identical to that of a single ip dose. On the other hand, the 3′,5′-dimyristoyl (fig 6) and 3′,5′-distearoyl (fig 7) derivatives are noticeably less effective by the sc route. With oral treatment, only very low activity was noted for the diesters, while 3′-myristoyl-cyclocytidine (fig 5) showed significant activity by this route at only very high doses.

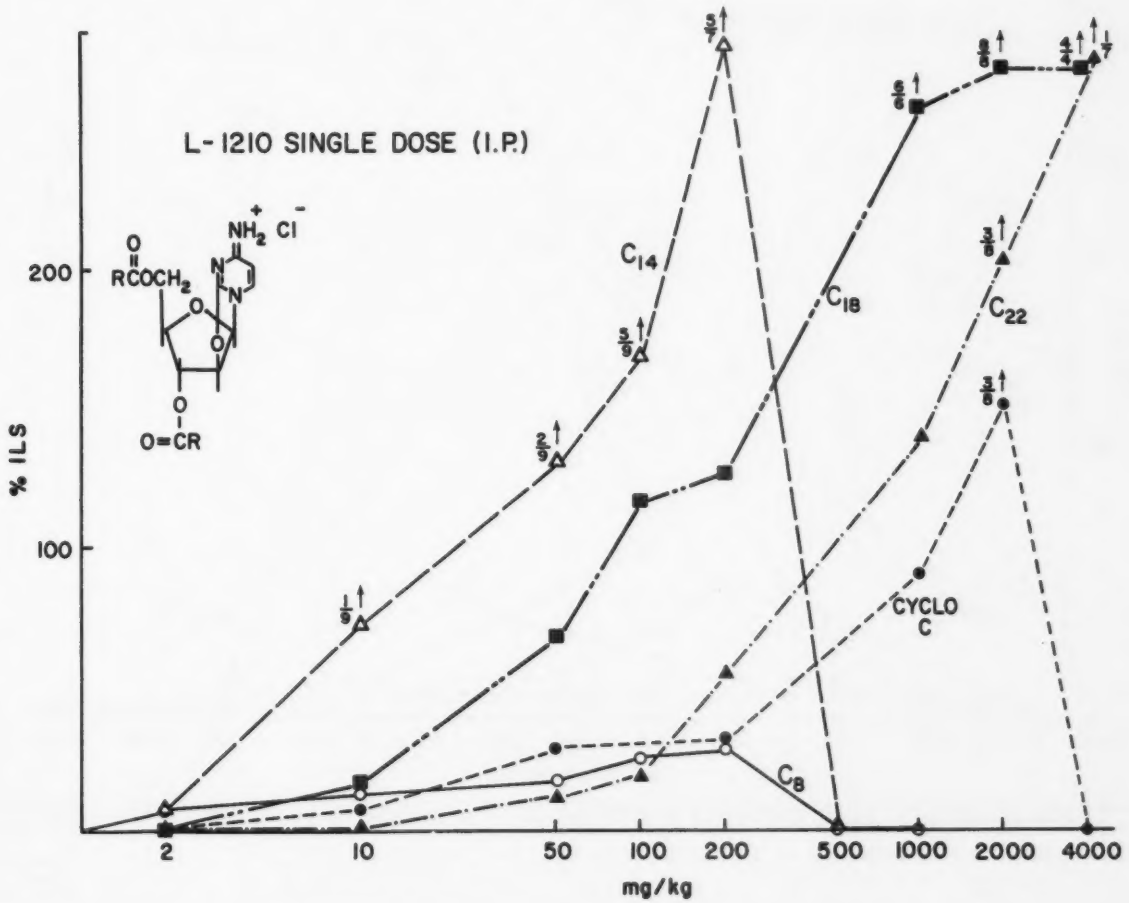


FIGURE 2.—Effects of single ip injections of 3′,5′-di-*O*-acyl-cyclocytidine hydrochlorides on mice bearing L1210 leukemia.

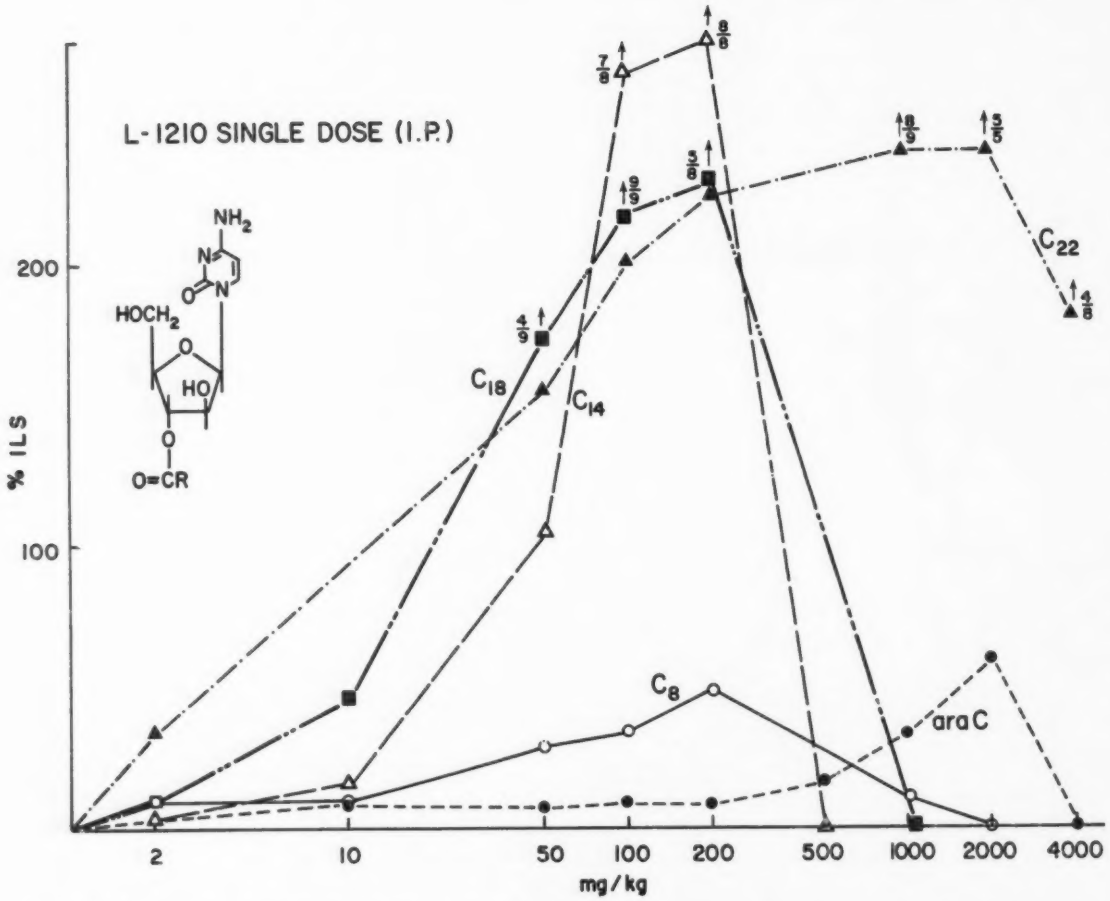


FIGURE 3.—Effects of single ip injections of 3'-*O*-acyl-cytosine arabinosides on mice bearing L1210 leukemia.

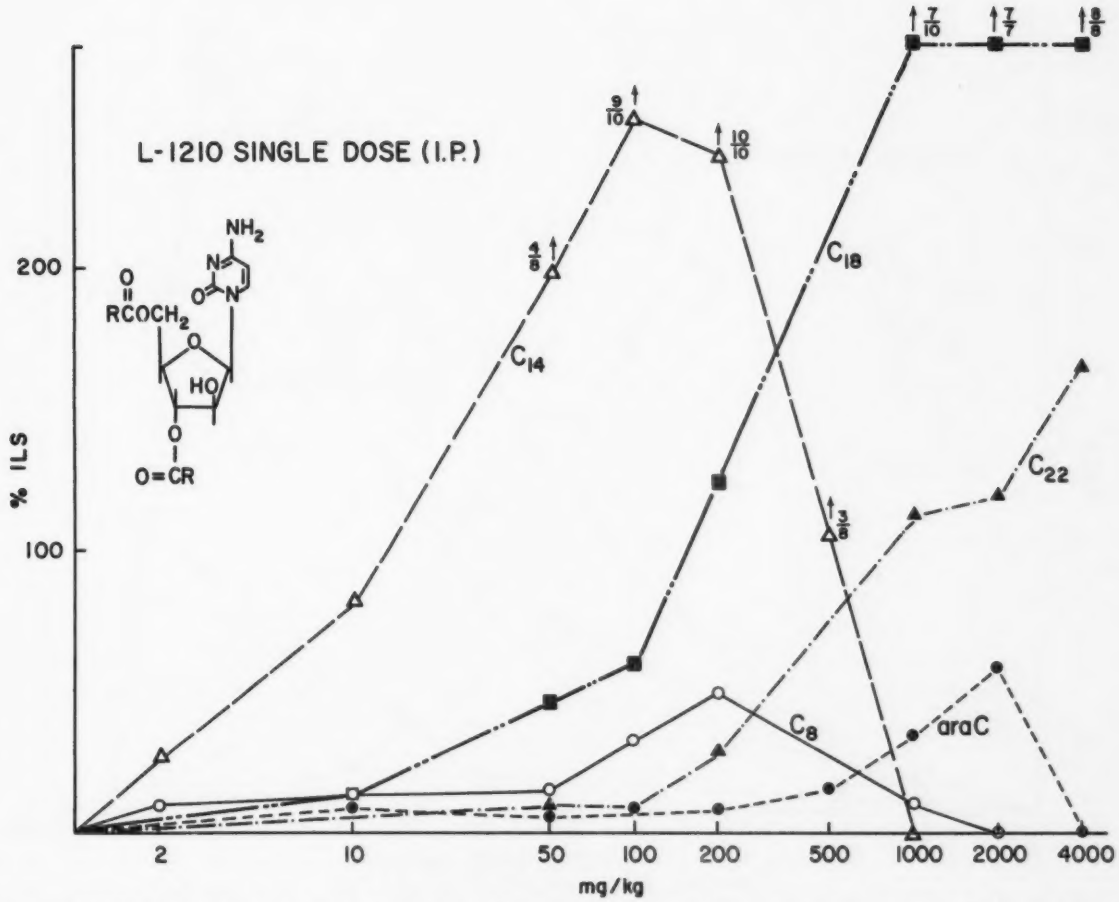


FIGURE 4.—Effects of single ip injections of 3′,5′-di-*O*-acyl-cytosine arabinosides on mice bearing L1210 leukemia.

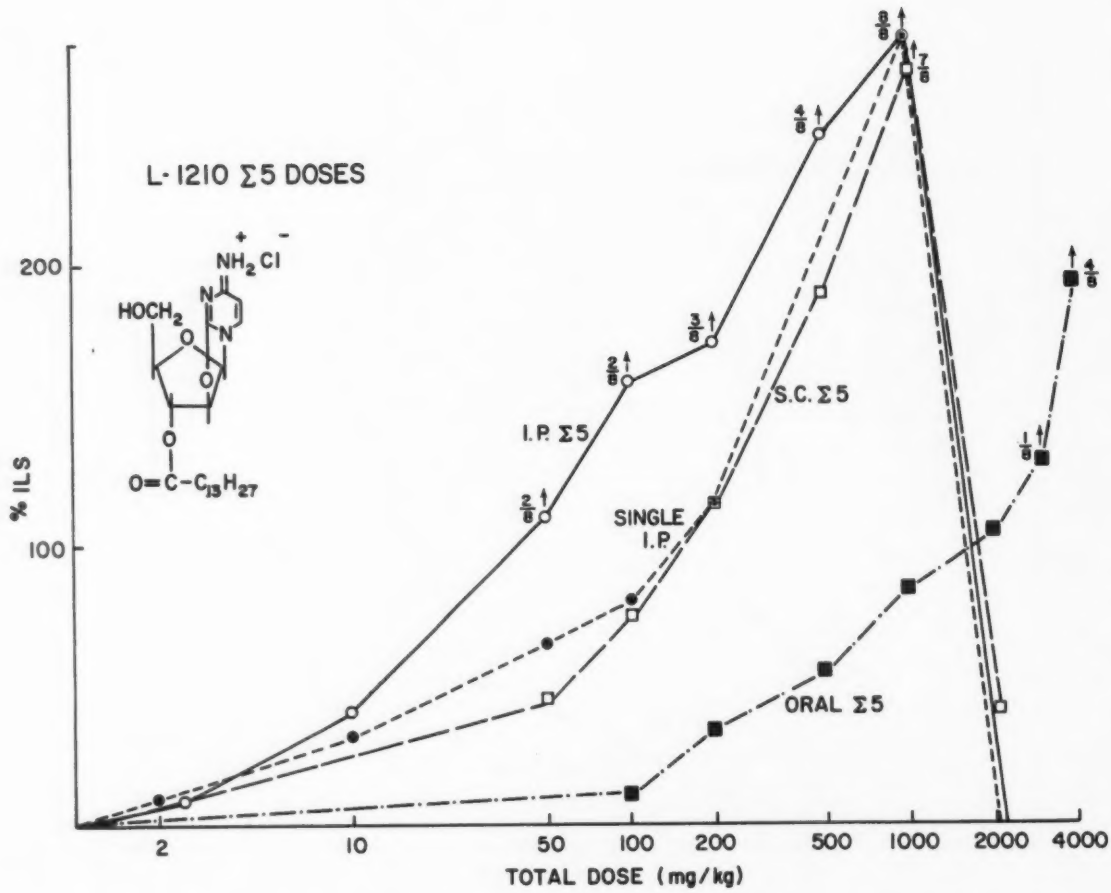


FIGURE 5.—A comparison of single and multiple administrations of 3′-*O*-myristoyl-cyclocytidine hydrochloride against L1210 leukemia in mice. The multiple doses (Σ5) refer to the sum of 5 equal doses on Days 1, 2, 3, 4, and 5 by the route shown.

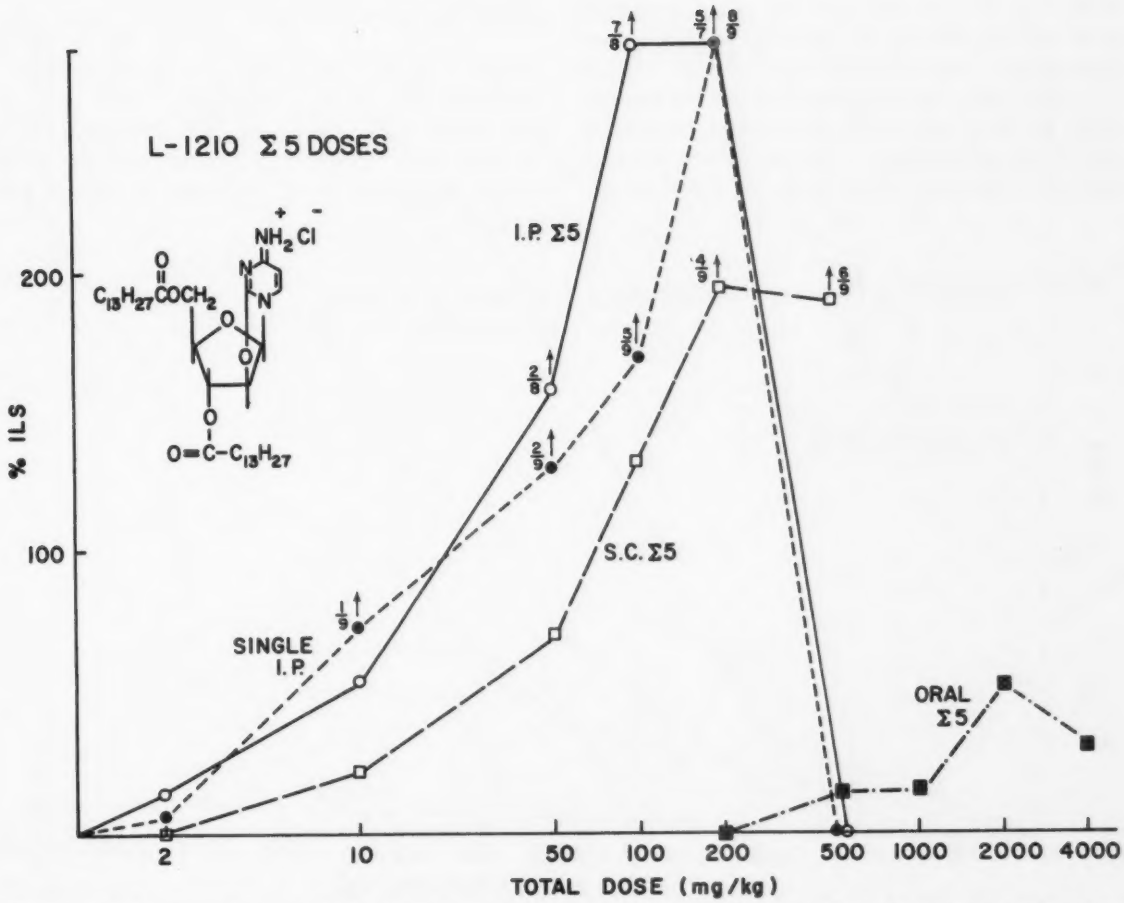


FIGURE 6.—A comparison of single ip and varied multiple administrations of 3′,5′-di-*O*-myristoyl-cyclocytidine hydrochloride.

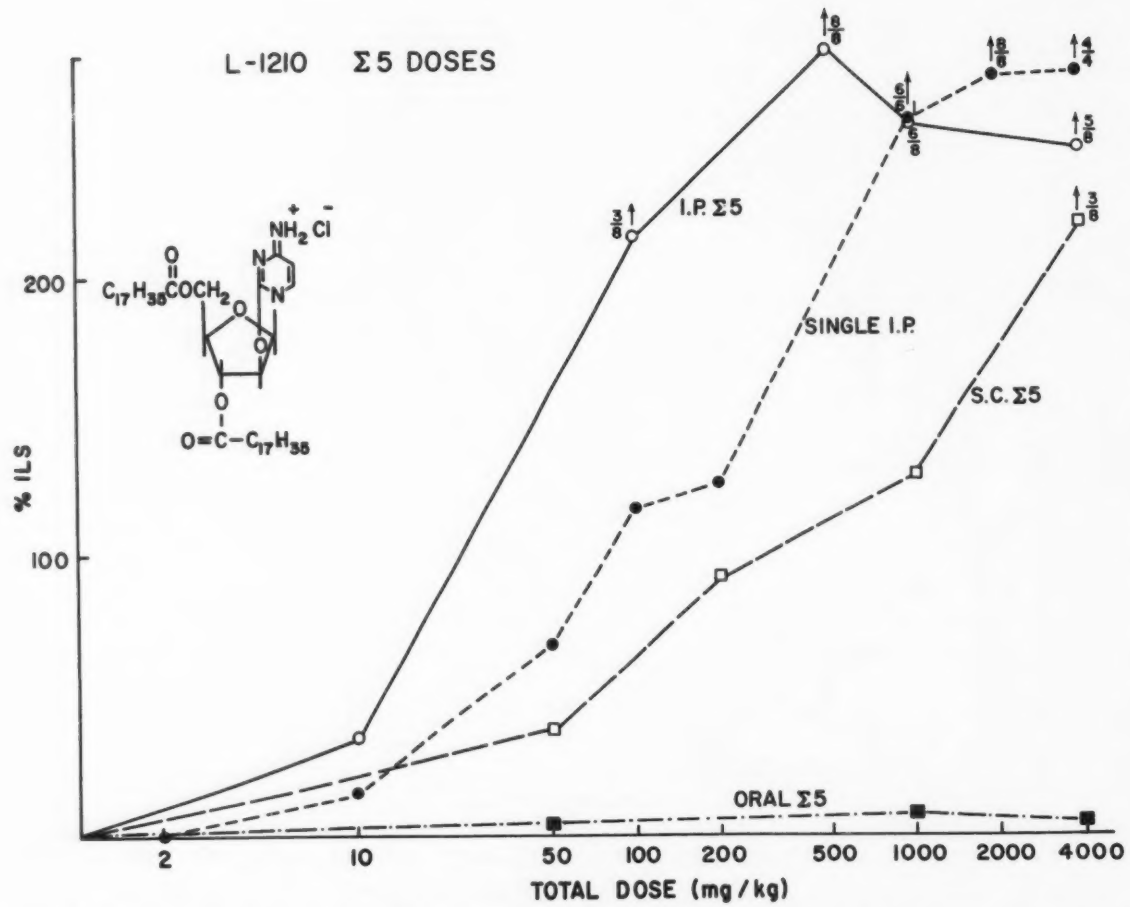


FIGURE 7.—A comparison of single ip and varied multiple (Σ5) administrations of 3',5'-di-*O*-stearoyl-cyclocytidine hydrochloride.

It should also be pointed out that certain of the above compounds show marked activity against advanced L1210 leukemia in mice. Thus, for example, 3′-myristoyl-cyclocytidine (*33*, R = $C_{13}H_{27}$) gave eight 30-day survivors among eight animals treated sc at a dose of 200 mg/kg/day on Days 5, 6, 7, 8, and 9 following infection. The action of the compounds was also very long lived since significant increases in lifespan were observed when the drug was administered sc as long as 10 days prior to inoculation of leukemic cells.

We have also examined the action of these various esters of cyclocytidine and cytosine arabinoside against DS strain mice implanted with Ehrlich ascites carcinoma. Once again, the necessary effective doses of the different compounds varied widely, but in figure 8 we compare the activities of the homologous series of types *33–36* at an arbitrary dose of 20 mg/kg/day administered ip on Days 1, 2, 3, and 4 following implantation. On Day 10 the animals were killed and a tumor index was determined from the total packed volume of the ascitic cells in the treated group divided by that from untreated controls (33). From figure 8 it can be seen that at the dose level chosen, cytosine arabinoside is considerably more active than cyclocytidine and that, unlike the results with L1210, the 3′-acyl-cyclocytidine compounds showed little significant activity. Also, the 3′,5′-diacylated cytosine arabinosides were of little interest except for the C_{18} (stearoyl) derivative. On the other hand, the 3′,5′-diacyl-cyclocytidine

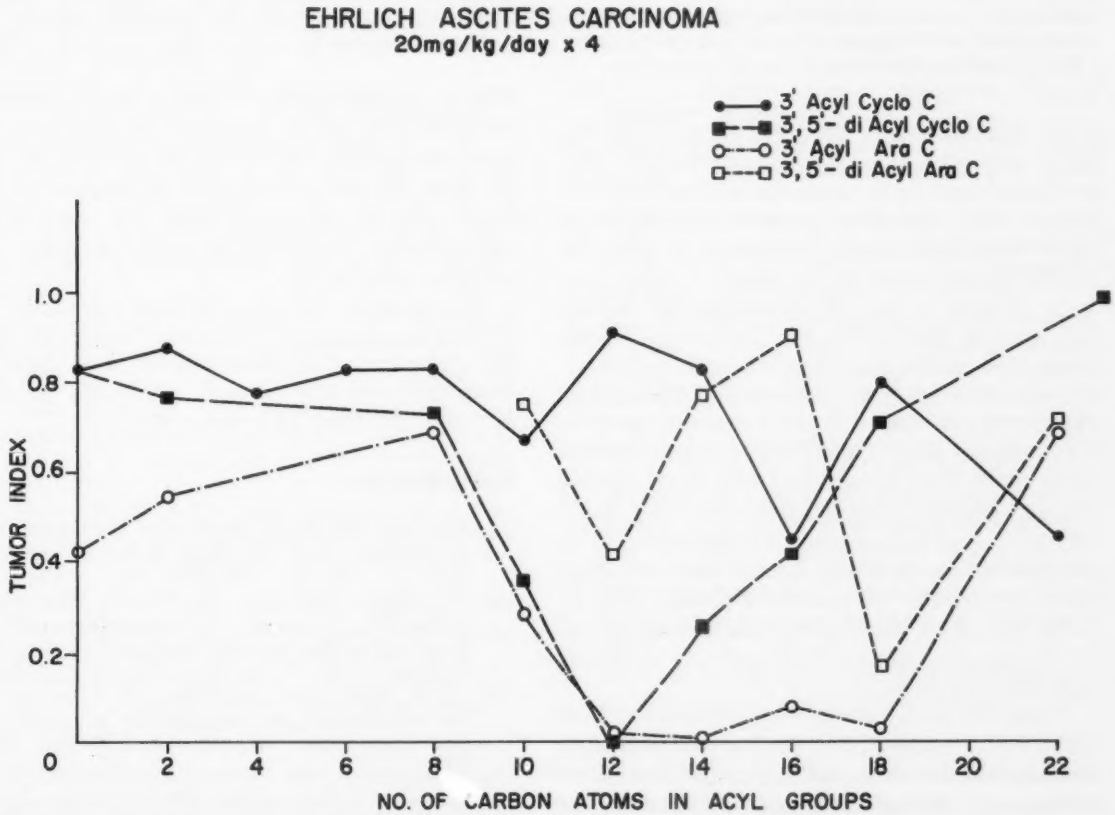


FIGURE 8.—A comparison of the effects of different 3′-*O*-acyl- and 3′,5′-di-*O*-acyl- derivatives of cyclocytidine and cytosine arabinoside administered ip on Days 1, 2, 3, and 4 to mice bearing Ehrlich ascites carcinoma. *See* text.

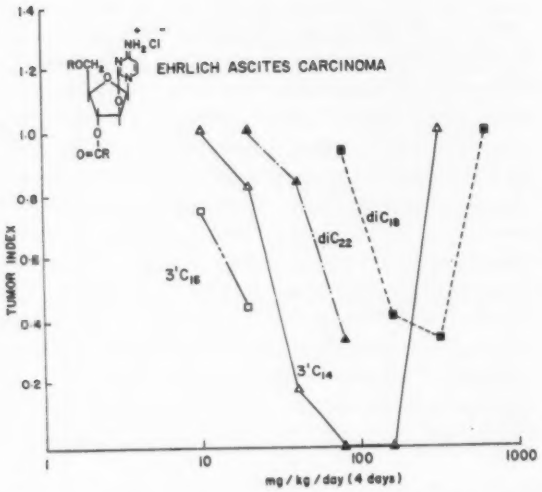


FIGURE 9.—Dose-response curves for certain 3'-*O*-acyl- and 3',5'-di-*O*-acyl-cyclocytidine hydrochlorides administered ip on Days 1, 2, 3, and 4 to mice bearing Ehrlich ascites carcinoma.

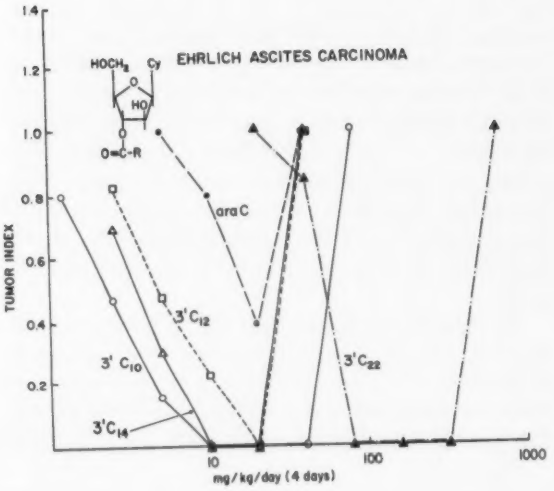


FIGURE 10.—Dose-response curves for certain 3'-*O*-acyl-cytosine arabinosides administered ip on Days 1, 2, 3, and 4 to mice bearing Ehrlich ascites carcinoma.

series showed a very pronounced maximal activity with the C_{12} (lauroyl) derivative, this compound leading to complete inhibition of the tumor cells. The 3'-acyl-cytosine arabinoside series was most generally efficacious, with the C_{12}–C_{18} homologs all being highly active.

In figures 9 and 10 dose-response curves for several derivatives are recorded. From figure 9 it can be seen that while 3'-myristoyl-cyclocytidine (C_{14}) is ineffective at 20 mg/kg/day it is highly active at an 80–200-mg/kg dose. As concluded above, the 3'-acyl-cytosine arabinoside series is apparently the most active against Ehrlich ascites carcinoma. The 3'-C_{10} and 3'-C_{14} derivatives show maximal activity at administration of 10–20 mg/kg/day but show signs of toxicity at 40–80-mg doses. The 3'-behenoyl (C_{22}) derivative, while inactive at 20 mg/kg/day, showed high activity at larger (80–320-mg) doses.

From the data presented in this paper it is clear that 3'- and 3', 5'-acylated derivatives of cytosine arabinoside and cyclocytidine show pronounced biologic activities relative to the parent nucleosides against both L1210 leukemia and Ehrlich ascites carcinoma. It is particularly noteworthy that the dose-response curves within an homologous series are frequently quite variable. Much work is necessary in order to optimize both the particular derivative to be used and the mode of administration. Detailed studies concerning both the compounds described herein and related derivatives will be described shortly.

The present work has been the result of a fruitful collaboration between two companies, the chemical synthesis being done at Syntex Research and the biologic screening at the Shionogi Research Laboratories.

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Metabolic Conditioning and Metabolic Actuation: Experimental Approaches to Cancer Chemotherapy Involving Combinations of Metabolites and Antimetabolites

A. Bloch [1]

SUMMARY

Two concepts involving metabolite-antimetabolite pairs are proposed which lend themselves to potential application in chemotherapy. One concept, termed *metabolic conditioning*, envisions the selective manipulation of the metabolism of the tumor or the host tissues by means of exogenously supplied metabolites, so as to achieve a more selective response to the action of an antitumor agent. The other concept, termed *metabolic actuation*, refers to the interference, by an antimetabolite, with the metabolic conversion of an exogenously supplied metabolite, causing it to accumulate in the cell and inciting it thereby to exert a regulatory effect on cell growth. Examples are provided to illustrate these concepts.

[Cancer Chemother Rep 58:471–477, 1974]

In the past, antimetabolites have been used extensively for the treatment of neoplastic diseases. However, only limited attention has been paid to the use of natural metabolites in combination with antimetabolites for inhibiting tumor growth or for achieving greater selectivity of drug action. A singular example of the successful clinical use of a metabolite-antimetabolite pair is provided by the combination of methotrexate [2] with folinic acid (N^5-formyl THFA), the metabolite being used to improve the selectivity of the drug (1). Because of such observations it appears that greater emphasis should be placed on the use of meaningful metabolite-antimetabolite combinations in chemotherapy. Based on our findings in experimental systems, we have formulated two new concepts which potentially lend themselves to chemotherapeutic exploitation.

One concept, termed *metabolic conditioning*, envisions the selective manipulation ("conditioning") of the metabolism of the tumor or the host tissues by means of exogenously supplied metabolites, so as to achieve a more selective response to the action of an antitumor agent.

The simplest example of this concept is that of a bacterial cell, wherein the concentration of a given enzyme, which is the target for an inhibitor, is altered by an exogenously supplied metabolite. For instance, we have shown (2) that the intracellular level of the dihydrofolate reductase of *Streptococcus faecium* is increased six- to ten-fold after the addition of 10^{-8}–10^{-10} M folic acid to the growth medium. As a result, the "induced" cells are approximately ten times less sensitive to inhibition of their growth by methotrexate than are the noninduced cells, the inducible enzyme being the primary target for methotrexate inhibition

[1] Department of Experimental Therapeutics and J. T. Grace, Jr. Cancer Drug Center, Roswell Park Memorial Institute, New York State Department of Health, Buffalo.

[2] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

(3). The use of *metabolic conditioning* to achieve greater selectivity is equally feasible in the intact animal. For example, 3-deazauridine,[3] an analog of uridine synthesized by Robins et al (4–7), increased the lifespan of male DBA_2/Ha mice bearing L1210 cells by approximately 50%–65% at intraperitoneal (ip) doses of 100–300 mg/kg/day × 6, and provided a dose-dependent number of 30-day survivors (8). However, the drug proved to be rather toxic to female mice and because of this toxicity caused only a 25% increase in the lifespan of female tumor-bearing mice. That this differential toxicity is sex-related was confirmed by the observation (9) that castrated female mice lost less weight (average loss, 3.4 g) as compared to intact controls (4.8 g), whereas the castrated males lost more weight (average, 3.5 g) than did the intact controls (1.9 g), so that the final response was about equal in both castrated sexes. Thus, it appeared logical to attempt to prevent the pronounced drug toxicity in the females through the administration of testosterone simultaneously with the drug. Under these conditions (fig 1), the extensive weight loss caused by 3-deazauridine was either completely eliminated or was extensively decreased, and, as a consequence, mortality was extensively reduced (9). The hormone also decreased the, albeit lesser, toxicity of the drug in the male mice. The same beneficial response was obtained when a depot form of the hormone, testosterone enanthate, was given 4 days prior to the drug. The hormone itself did not affect the growth of the tumor or its response to the action of the drug. Thus, *metabolic conditioning* was achieved.

[3] 3-Deazauridine: NSC-126849; CAS reg. No. 23205-42-7; 2,4(1*H*,3*H*) pyridinedione, 1-β-D-ribofuranosyl-.

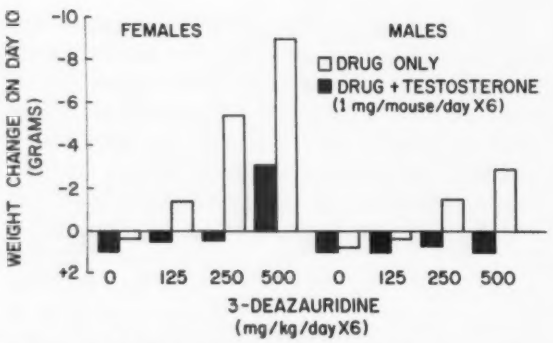

FIGURE 1.—Effect of testosterone on 3-deazauridine toxicity in DBA_2/Ha mice. The drug was given ip concomitantly with sc administered testosterone.

The alleviation of drug toxicity by testosterone appears to be due mainly to an effect of the hormone on the intestinal epithelium of the mouse. Histopathologic examination of the drug-treated animals showed that the drug toxicity was directed primarily against the intestinal epithelium. The duodenal crypts of female animals treated with the drug showed a marked decrease in mitotic activity and the presence of many pyknotic cells. Testosterone modified these effects, allowing extensive mitotic activity and decreasing the pronounced necrosis caused by the drug. Similarly, drug treatment led to atrophy of the intestinal villi, which appeared normally elongated and had characteristically columnar lining cells when testosterone was provided concomitantly with the drug (fig 2). It remains to be determined by what molecular mechanism testosterone prevents the drug toxicity to the intestinal epithelium. Testosterone given subcutaneously (sc) 5 hours before the ip administration of radioactive uridine, thymidine, and leucine (10 microcuries of each) caused a 1.5–2.6-fold increase over control in the incorporation of these precursors into the RNA, DNA, and protein respectively of the intestinal epithelium of female mice. This observation might indicate that this anabolic hormone causes a general increase in the metabolic activity of the intestinal epithelium, effected by an increase in the concentration of some enzymes, one of which may be the target for 3-deazauridine or its phosphorylated derivatives. An approximately two- to three-fold increase in the concentration of such an enzyme could readily account for the observed decrease in toxicity.

Another approach by which a metabolite can interact with an inhibitor to affect cell growth is exemplified by a process we term *metabolic actuation.* As has been documented extensively both in bacterial and mammalian cells (10), metabolites can regulate cellular activity not only by affecting the intracellular levels of enzymes, but also by determining the extent of the activity of an enzyme by such processes as

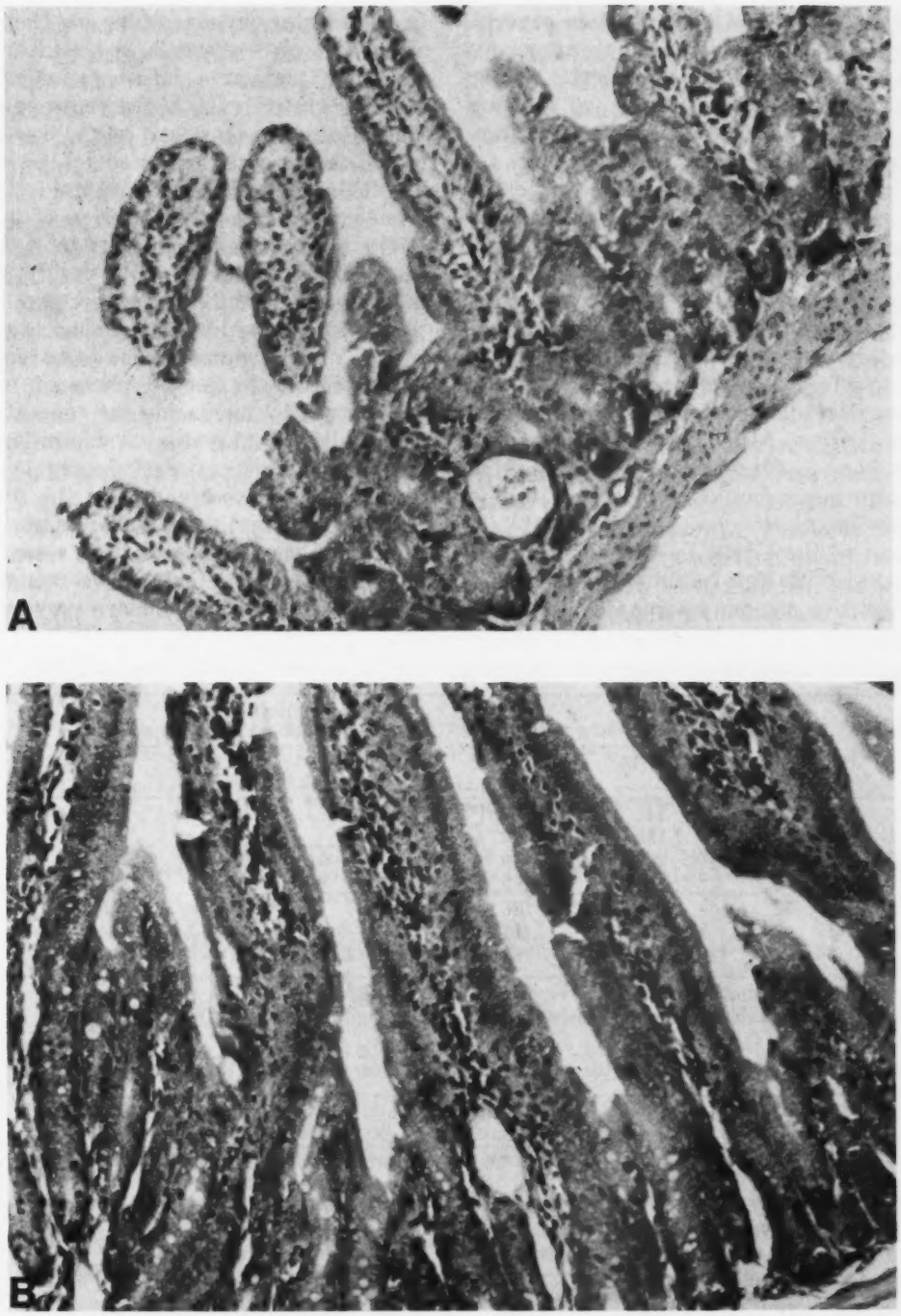

FIGURE 2.—Duodenal sections from female DBA_2/Ha mice treated for 4 days with (*A*) 250 mg/kg/day of 3-deazauridine or (*B*) 250 mg/kg/day of 3-deazauridine and 1 mg/day of testosterone. Drug treatment (*A*) leads to shortening of the intestinal villi and dilation of some crypts. Simultaneous administration of testosterone (*B*) prevents these effects. (Hematoxylin and eosin, × 200.)

feedback inhibition and activation. In general, cells dispose of even relatively high exogenous concentrations of metabolites efficiently, either by metabolizing them rapidly or by limiting their uptake, without experiencing inhibition of their growth. The concept envisions the accumulation of high concentrations of an exogenously supplied metabolite in the cell, caused by the presence of an analog which interferes with its metabolism. By virtue of its high concentration, the metabolite is "actuated" (defined as "to move to action," Webster) to exert a regulatory effect, which manifests itself through an effect upon cell growth.

The simplest illustration of the concept of *metabolic actuation* is the interaction between 9-(β-D-ribofuranosyl)-6-mercaptopurine (MPR) and guanosine (GR) observed in the *S. faecium* model system (11). MPR inhibits the growth of these cells by 50% at approximately 2×10^{-5} M. This inhibition is prevented in a competitive manner by guanine (table 1) or by adenine, hypoxanthine, or their nucleosides. GR also prevents the inhibition competitively, and, at a concentration of 10^{-5} M, restores growth even in the presence of 10^{-3} M MPR (table 2). However, as the concentration of GR is increased, renewed inhibition of the growth of the culture occurs (table 2). In the absence of MPR, this inhibition of growth by GR is not seen (bottom line, table 2). Thus, the interaction between MPR and GR gives rise to a biphasic inhibition of growth. The first phase, that caused by the analog, is prevented by the natural purines, but these metabolites do not affect the second phase of inhibition, that caused by increasing the concentration of GR. This second phase of inhibition is prevented by the natural pyrimidines; the pyrimidines, do not, however, affect the first phase of inhibition, that preventable by purines. Full growth of the culture at high concentrations of both MPR and GR can be restored when a pyrimidine and a purine are supplied jointly.

TABLE 1.—Effect of guanine on the in vitro growth of *S. faecium* cells in the presence of MPR *

MPR (M)	Control	Guanine (M) 1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
		(extent of growth in Klett units †)					
1×10^{-3}	0	141	52	0	0	0	0
1×10^{-4}	0	188	96	44	0	0	0
1×10^{-5}	0	220	148	73	36	0	0
1×10^{-6}	0	228	182	84	59	36	0
1×10^{-7}	23	230	186	86	64	55	40
1×10^{-8}	49	232	187	88	69	64	59
0	64	234	187	87	71	65	64

* The assay was carried out by procedures reported previously (2).
† Klett nephelometric values = optical density divided by 2 and multiplied by 1000.

TABLE 2.—Effect of GR on the in vitro growth of *S. faecium* cells in the presence of MPR *

MPR (M)	Control	GR (M) 1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
		(extent of growth in Klett units †)					
1×10^{-3}	0	0	0	49	0	0	0
1×10^{-4}	0	0	0	76	14	0	0
1×10^{-5}	0	0	67	84	29	14	0
1×10^{-6}	0	127	132	87	54	37	12
1×10^{-7}	16	182	172	85	60	54	33
1×10^{-8}	36	219	183	88	66	63	59
0	64	230	186	87	66	65	64

* The assay was carried out by procedures reported previously (2).
† Klett nephelometric values = optical density divided by 2 and multiplied by 1000.

Whereas the inhibition of de novo purine synthesis by 6-mercaptopurine[4] (and thereby by MPR) has been well documented (12) and explains why the first phase of inhibition can be prevented by supplying purines exogenously, the nature of the second phase of inhibition requires interpretation. As shown in table 3, a pronounced decrease in the conversion of GR to the ribonucleotide stage occurs in cell-free extracts in the presence of MPR. As a consequence, the concentration of GR increases in the cell. GR accumulation in the presence of the analog is also seen in the intact cells (table 4). As is shown in table 5, and as has been shown in mammalian cell extracts (13,14), GR is a feedback effector, inhibiting the activity of carbamylaspartate transferase and dihydroorotase, enzymes involved in de novo pyrimidine synthesis. The lack of pyrimidines caused thereby can be overcome by supplying these metabolites exogenously to the cells, in the same way as the lack of purines caused by 6-mercaptopurine is overcome by supplying purines. The observation that the concentration of MPR is also increased to some extent in the presence of GR (table 3) and that MPR itself exerts a feedback effect (table 5) would indicate that "actuation" extends to both the metabolite and antimetabolite, which interfere with each other's metabolism reciprocally.

The alleviation of the cytotoxicity of MPR at one concentration of GR and its enhancement by higher concentrations of the metabolite are also seen in Ehrlich ascites carcinoma cells in vivo (table 6), indicating that the approach is equally applicable to tumor cells.

The practical utility of the concepts presented in the chemotherapy of tumors requires, of course, extensive evaluation. Since the approaches are applicable to any metabolite-antimetabolite pair which possesses the potential for regulatory control, many additional combinations can be formulated. A selective effect of such combinations could arise from a variety of factors, including a differential response to feedback inhibition or a difference in

[4] 6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate.

TABLE 3.—Metabolism of GR and MPR and their combinations in cell-free extracts of *S. faecium**

Ribonucleoside added to incubation mixture	Base	Ribonucleoside	Nucleotides
	(amount present after incubation as % of radioactive compound added)		
GR (1×10^{-4} M)	1	5	82
GR (1×10^{-4} M) + MPR (1×10^{-4} M)	12	56	19
MPR (1×10^{-4} M)	9	20	58
MPR (1×10^{-4} M) + GR (1×10^{-4} M)	13	35	41

* The reaction mixture, in a total volume of 0.30 ml of 0.1 M phosphate buffer (pH 7.0), contained ATP and PRPP each at 5×10^{-3} M, 0.05 ml of the crude cell-free extract, and guanosine-8-^{14}C (1 microcurie, 46 millicuries/mmole) or 6-mercaptopurine ribonucleoside-^{35}S (1 microcurie, 26 millicuries/mmole) which were added to the respective nonlabeled compounds to the final concentration shown. Following incubation for 30 min at 37 C, the incubation mixtures were immersed in a boiling water bath for 2 min and centrifuged, and the supernatant solutions were applied to Whatman 3 MM paper and were chromatographed together with authentic markers (including GMP, GDP, GTP, and MPRP) in 1% aqueous H_3BO_3 buffer and in isoamyl alcohol-5% aqueous sodium citrate (1:1, v/v). The radioactivity of the products was determined in a scintillation spectrometer.

TABLE 4.—Metabolism of GR by intact *S. faecium* cells in the presence of MPR *

Additions to growth medium	Guanine (amount present in the acid-soluble fraction as % of total radioactivity in the fraction)	GR	Guanine nucleotides
GR (1×10^{-4} M)	12	8	73
GR (1×10^{-4} M) + MPR (1×10^{-3} M)	7	29	54

* The organism was grown for 18 hrs in 1 liter of culture medium (15). Following centrifugation, the cells were resuspended in 360 ml of fresh medium and were incubated at 37 C for 90 min. The culture was then divided into two portions, GR being added to one, GR and MPR to the other. Guanosine-8-^{14}C (10 microcuries, 46 millicuries/mmole) was added to the nonlabeled nucleoside to give the final concentration shown. The cultures were incubated for 60 min at 37 C, and following centrifugation the pellets were extracted with 10% perchloric acid and were neutralized with 0.2N KOH. The extracts were reduced in volume in vacuo, and aliquots were applied to Whatman 3 MM paper together with authentic markers (including GMP, GDP, and GTP). They were then chromatographed in *n*-butanol-50% aqueous acetic acid (1:1, v/v) and in *n*-propanol-water (60:40, v/v). The radioactivity of the appropriate products was determined in a liquid scintillation spectrometer. The amount of labeled nucleoside remaining in the medium after incubation was also determined, and the amount present in the intercellular water of the pellet was subtracted from the concentration found in the pellet extract (15).

TABLE 5.—Effect of GR and MPR on de novo pyrimidine synthesis in cell-free extracts of *S. faecium**

Ribonucleoside added to the incubation mixture	Aspartate-^{14}C converted to dihydroorotate and orotate † %
None	42
MPR (3×10^{-4} M)	28
GR (3×10^{-4} M)	23
GR (3×10^{-4} M) + MPR (3×10^{-3} M)	19

* The reaction mixture, in 0.75 ml of 0.2 M tris buffer (pH 9.2), contained L-aspartic acid-^{14}C (160 millicuries/mmole), 0.1 μmole; carbamyl phosphate, 0.4μmole; and 0.5 ml of the crude cell-free extract. Incubation proceeded for 30 min at 37 C, and the reaction was stopped by the addition of 0.2 ml of 4N perchloric acid. Following centrifugation, authentic carriers were added to the mixtures, the products of the reaction were separated by electrophoresis in 0.04 M citrate buffer (pH 2.5), and their radioactivity was determined in a liquid scintillation spectrometer.

† The labeled material remaining was present essentially as aspartic or carbamylaspartic acid.

TABLE 6.—Effect of MPR-GR combinations on the in vivo growth of Ehrlich ascites carcinoma cells *

Treatment	Dose (mg/kg/day × 7)	Cell number (10^6) on Day 8
Saline	—	541 ± 53
GR	50	553 ± 72
GR	100	698 ± 92
MPR	100	265 ± 37 †
MPR + GR	100 + 50	344 ± 81 †
MPR + GR	100 + 100	123 ± 40

* On Day 0, 6–8-week-old female Ha/ICR Swiss mice were inoculated ip with 1×10^6 tumor cells. Treatment was begun on Day 1 and the compounds were administered ip. The cells were removed into saline on Day 8 and were counted with a standard hemocytometer. The data shown are averages from 3 experiments with 5 mice/group/experiment.

† The difference between these 2 groups is statistically significant at $0.05 > P > 0.025$.

the extent of uptake or metabolic disposition of the combinants by the tumor as compared to the normal tissues. These possibilities deserve further attention.

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Mechanisms of Action of Antitumor Antibiotic Inhibitors of Protein Synthesis [1]

Irving H. Goldberg [2]

SUMMARY

The current state of information on the mechanism of action of two antitumor antibiotics that inhibit mammalian protein synthesis at separate and discrete steps will be discussed. These agents have proved to be useful tools in the study of the biochemistry and physiology of protein synthesis and its regulation in mammalian cells. Because of considerations of general cytotoxicity probably due to lack of selectivity for growing neoplastic cells, these drugs, in contrast to nucleic acid synthesis inhibitors, have not yet proved to be of clinical value in man. Hopefully, a greater understanding of the events in cell growth that are dependent on new and continuing protein biosynthesis will lead to the availability of agents that have a selective toxicity for neoplastic cells.

[Cancer Chemother Rep 58:479–489, 1974]

Having recently reviewed in some detail the mechanisms of action of antitumor antibiotics that inhibit cell growth by interfering with nucleic acid synthesis or function (1), I shall present here a progress report of our work on the basis of action of two antibiotic inhibitors (sparsomycin and pactamycin [3] [fig 1]) (2,3) of ribosomal protein synthesis in eukaryotes. While toxic effects have limited their use in the treatment of neoplasia in man (4,5), these agents have proved to be of extraordinary value as tools in the study of the normal events in protein synthesis. Thus, sparsomycin has been used to block selectively and specifically the peptidyl transferase located on the larger ribosomal subunit, and pactamycin, as a specific inhibitor of polypeptide-chain initiation, has been used to determine the gene order of mammalian cell viruses (6–8) and to show that both completed α-globyl-tRNA and β-globyl-tRNA are normal intermediates of soluble hemoglobin biosynthesis (9). Both antibiotics have been found to be most cytotoxic to tumor cells in the S phase of the cell cycle (10).

SPARSOMYCIN AND THE PEPTIDYL TRANSFERASE

Sparsomycin blocks the function of the enzyme system, located on the larger ribosomal subunit (60S), that is responsible for peptide-bond formation. A convenient way to study this reaction in isolation from the other steps

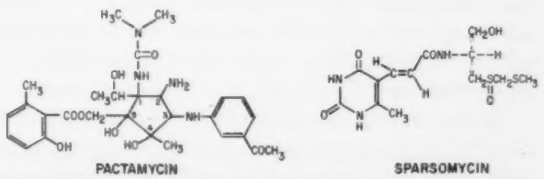

FIGURE 1.—Structures of pactamycin and sparsomycin.

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[2] Department of Pharmacology, Harvard Medical School, Boston, Mass.

[3] Sparsomycin: NSC-59729; CAS reg. No. 1404-64-4. Pactamycin: NSC-52947; CAS reg. No. 23668-11-3.

involved in protein synthesis is by the use of another antibiotic, puromycin,[4] an analog of the aminoacyladenosine end of tRNA. The nascent peptide chain in the P- or donor-site on the ribosome is transferred to the amino group of the puromycin molecule in a reaction catalyzed by the ribosome-bound peptidyl transferase (11). This reaction, which results in the release of the nascent peptide from the ribosome as peptidylpuromycin, is readily blocked by sparsomycin (12,13). Further, sparsomycin is a competitive inhibitor of puromycin in the puromycin-induced release of polylysine bound to ribosomes (fig 2). On the other hand, in an incubation in which the sparsomycin-sensitive formation of single peptide bonds was measured by the addition of a single labeled lysine residue onto the end of an existing polylysine chain attached to the P-site of the ribosome sparsomycin, inhibition could not be overcome by increasing the concentration of lysyl-tRNA for which puromycin serves as an analog (14). Since aminoacyl-tRNA depends primarily on codon recognition and interaction with the smaller ribosomal subunit for its binding to the ribosome (whereas puromycin interaction takes place at another location in the A-site on the ribosome, probably at the peptidyl transferase itself), it is not surprising that sparsomycin competes with one and not the other. These data suggested that sparsomycin acts at or close to the actual site where peptide-bond formation takes place, the peptidyl transferase. In support of this conclusion are other experiments in which the formation of a single peptide bond has been studied isolated from the other steps involved in polypeptide synthesis. In the so-called fragment reaction, an *N*-substituted aminoacyl or peptidyl moiety attached to a short fragment of tRNA can be transferred to puromycin or another acceptor in the presence of only the larger ribosomal subunit (or its subparticle) containing the peptidyl transferase and in the absence of the smaller ribosomal subunit and other factors. This reaction is sensitive to sparsomycin (15).

Sparsomycin has another effect that is probably related to its interaction with the ribosomal peptidyl transferase. Sparsomycin appears to "freeze" the initiator-tRNA or the peptidyl-tRNA in the P-site on the ribosome (16). As shown in figures 3 and 4, low concentrations of sparsomycin stimulate the overall binding of initiator- (or peptidyl-) tRNA to ribosomes, in which form it cannot be released by puromycin. In these experiments, the binding of *N*-acetylphenylalanyl-tRNA to ribosomes was followed with polyuridylic acid as the mRNA. This freezing effect of sparsomycin is also found in the fragment reaction (17). By contrast, the binding to ribosomes of the aminoacyl-tRNA fragment, in which the amino group of the amino acid is unsubstituted, is inhibited by sparsomycin (18). These data then suggest that sparsomycin interferes with the "acceptor" recognition area in the peptidyl transferase (on the larger ribo-

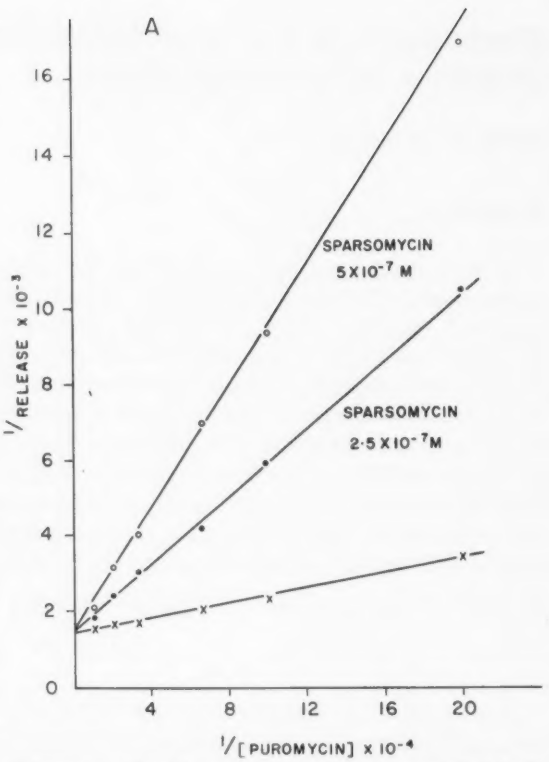

FIGURE 2.—Double reciprocal plot of sparsomycin inhibition of the puromycin reaction (13).

[4] Puromycin: NSC-3055; CAS reg. No. 58-58-2; adenosine, 3'-(α-amino-*p*-methoxyhydrocinnamamido)-3'-deoxy-*N*,*N*-dimethyl-, dihydrochloride.

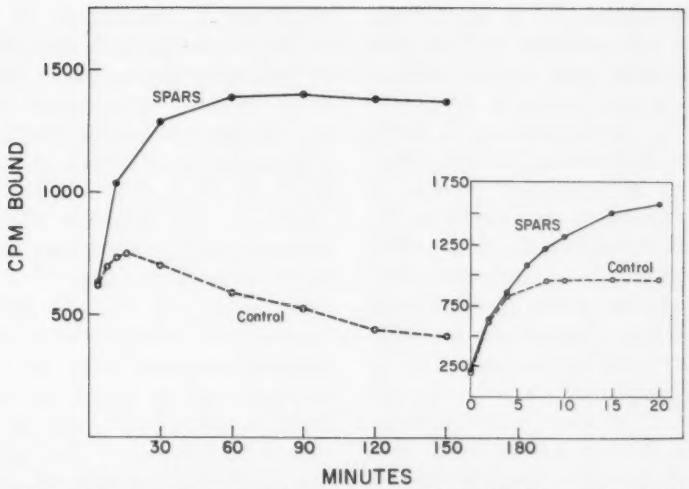

FIGURE 3.—Sparsomycin stimulation of polyuridylate-promoted binding of ^{14}C-*N*-acetylphenylalanyl-tRNA to ribosomes. Sparsomycin (10^{-4} M) was added at the start of the reaction as indicated. (*See* ref 16 for experimental details.)

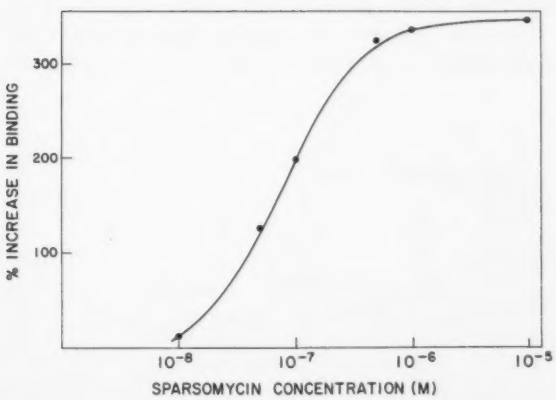

FIGURE 4.—Relation of sparsomycin concentration to the stimulation of binding of *N*-acetylphenylalanyl-tRNA to ribosomes. Results are expressed as percent stimulation of binding found in the absence of antibiotics. (*See* ref 16 for experimental details.)

somal subunit), thereby blocking interaction with the aminoacyl end of an incoming aminoacyl-tRNA (or with the amino group of puromycin) and fixing the peptidyl-tRNA in the donor site. Such an action differs from that of tetracycline which blocks codon recognition between the tRNA portion of the aminoacyl-tRNA and the mRNA-ribosome complex (probably on the smaller ribosomal subunit) (19, 20).

PACTAMYCIN AND INITIATION

In bacterial extracts it was found that in the presence of pactamycin the initiation complex possessed an altered structure with decreased stability at low magnesium concentrations (21–23). Further, in polyphenylalanine synthesis at low magnesium concentrations where *N*-acetylphenylalanine-tRNA served as the initiator-tRNA, pactamycin appeared to have a primary effect on polypeptide initiation. Pactamycin does not affect peptide-bond formation as such, since the puromycin reaction with prebound peptide, or its equivalent, in the P-site is not inhibited by this agent (23).

Strongest support for a primary action of pactamycin on polypeptide chain initiation comes from experiments using intact cells and extracts from mammalian sources (24–27). As shown in figure 5, two cell-free globin-synthesizing systems derived from rabbit reticulocytes, which differ in their ability to start new peptide chains, were compared for their sensitivity to different concentrations of pactamycin. Comparison of A and B reveals that

polypeptide synthesis in the lysate system, in which over 70% of the globin made is due to the formation of new chains (B), is considerably more sensitive to inhibition by pactamycin than is that in the fractionated system where less than 10% can be so accounted for (A). Further, inhibition of incorporation is seen after a lag of about 2 minutes; during that time chain elongation is completed (and it is the time required to translate the globin message). As was found by Colombo et al (28) with intact reticulocytes, in lysates that are able to initiate new chains, a low level of pactamycin (about 10^{-6}M, depending on the amount of ribosomes) leads to the rapid (within 2 minutes) and sequential breakdown of larger polyribosomes to smaller polyribosomes and finally to single 80S ribosomes with the release of completed α- and β-globin chains (fig 6). The latter have been identified by polyacrylamide gel electrophoresis. Ribosomal subunits are not produced by pactamycin. At higher concentrations of pactamycin (10^{-5}M) polyribosome breakdown is incomplete and globin release is slowed. These data suggest that at low concentrations of pactamycin, initiation of protein synthesis is affected much more than elongation or termination, but at the higher concentrations elongation may be affected as well. By interfering primarily with the initiation step, pactamycin prevents the formation of new polyribosomes while allowing for read-off of mRNA and runoff of ribosomes by existing ones. Antibiotic inhibitors of peptide-chain elongation, such as sparsomycin, cycloheximide, or fusidic acid,[5] prevent the pactamycin-induced polyribosome breakdown. While polyribosome decay and radioactive peptide release are completely expressed within 2 minutes of the addition of 3.5×10^{-6}M pactamycin, there appeared to be a dissociation between these two parameters at the 30-second point (fig 7). The radioactivity, which persists on the polyribosomes for the first 30 seconds, results from the continued addition of radioactive amino acids onto elongating peptide, as shown by the fact that the addition of excess unlabeled amino acids obliterates the lag in the radioactivity release from the polyribosomes. The continued incorporation of radioactive amino acids associated with elongation in the absence of initiation also accounts for the slightly more than twofold increase in labeled protein released into the soluble fraction in the presence of pactamycin. Further, as expected of an agent that predominantly blocks polypeptide-chain initiation, the patterns of release of radioactivity from polyribosomes and of accumulation in the supernatant fraction are practically identical with the control when further incorporation of radioactivity is prevented by excess unlabeled amino acids. The fact that labeled nascent peptide is better maintained on the polyribosomes at 30 seconds than the polyribosomes themselves is further evidence against the premature release of incomplete polypeptide chains due to pactamycin.

Additional evidence supporting an action of pactamycin on initiation is provided by experiments in which polypeptide synthesis stimulated by a preparation of initiation factors (KCl wash) is found to be much more sensitive to inhibition by the antibiotic than is synthesis

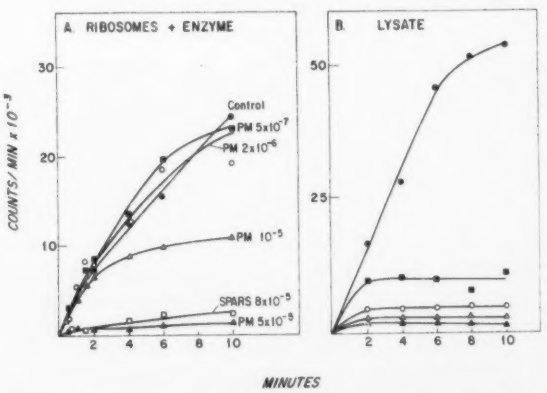

FIGURE 5.—Effect of pactamycin on protein synthesis in fractionated (A) and whole (B) lysates from rabbit reticulocytes. On the ordinate is plotted the incorporation of ^{14}C-valine into polypeptide. NH_2-terminal ^{14}C-valine analysis of the synthesized globin revealed that less than 10% of the globin made in A is due to the formation of new chains, whereas over 70% of that made in B is synthesized de novo. PM = pactamycin; SPARS = sparsomycin. (*See* ref 25 for details.)

[5] Cycloheximide: NSC-185; CAS reg. No. 7006-21-5; glutarimide, 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-.

Fusidic acid: NSC-56192; CAS reg. No. 62-60-2.

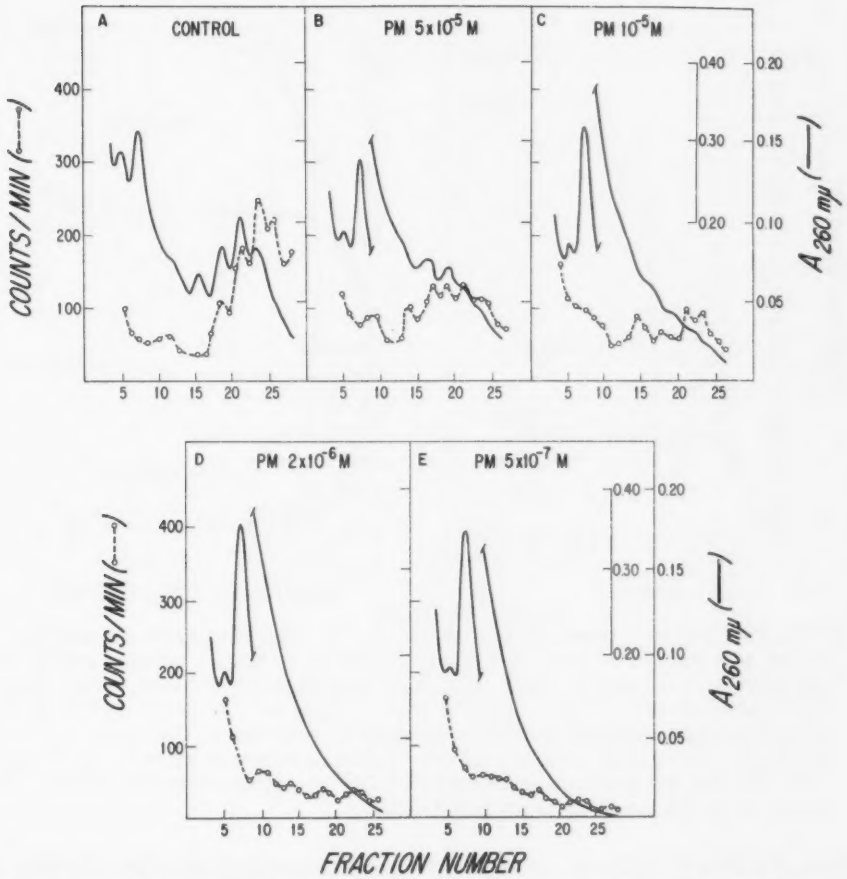

FIGURE 6.—Effect of pactamycin concentration on the breakdown of polyribosomes and release of peptide in rabbit reticulocyte lysates. Polyribosomes prelabeled with ^{14}C-amino acids in lysates were then incubated for 2.25 min with different pactamycin concentrations. An aliquot of the reaction was analyzed by sucrose density gradient centrifugation (from left to right: the main single peak at $A_{260m\mu}$ is at 80S [25]).

that takes place in the absence of added initiation factors (table 1). In the former, new globin chains are being made, while in the latter, incorporation of radioactive amino acid represents mainly completion of existing nascent chains. This differential effect of pactamycin on initiating and noninitiating systems is not found with antibiotics such as chlortetracycline [6] and sparsomycin which affect only elongation.

Pactamycin binds rapidly to the smaller (30S) ribosomal subunit from bacteria (22) and also interacts with the smaller (40S) ribosomal subunit from mammalian cells (24). At 0 C, pactamycin binds to the rabbit reticulocyte 40S ribosomal subunit, as well as to the 80S ribosome, but not to polyribosomes (fig 8A). The binding to 80S ribosomes is presumably by way of the smaller subunit, since there is no binding to the isolated larger subunit. Further, binding to the free smaller subunit has the highest specific activity. Single ribosomes derived from polyribosomes by ribo-

[6] Chlortetracycline: NSC-13252; CAS reg. No. 64-72-2.

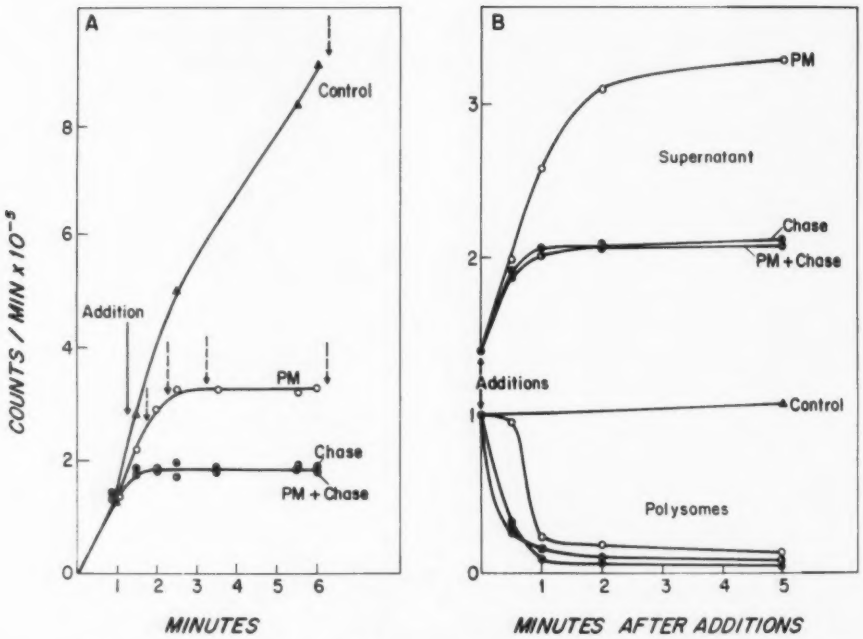

FIGURE 7.—Effect of pactamycin (3.5×10^{-6} M) on polypeptide synthesis and distribution as a function of time. At 1.25 min (additions, A and B) solutions containing pactamycin, pactamycin + chase (containing large excess of unlabeled amino acids), and a chase solution without pactamycin were added as indicated to 3 incubations of reticulocyte lysates synthesizing polypeptide as measured by the incorporation of radioactive leucine and valine. At the times indicated by broken arrows in A, aliquots were removed and fractionated by centrifugation into supernatant and polyribosome fractions B. (*See* ref 25 for experimental details.)

TABLE 1.—Effects of different antibiotics on the KCl wash-stimulated incorporation of amino acids into protein (29)

Antibiotic	M	Inhibition (%) Without KCl wash	Inhibition (%) With KCl wash
Pactamycin	1×10^{-6}	0	70
Chlortetracycline	1×10^{-4}	27	19
	2×10^{-4}	42	42
Sparsomycin	1×10^{-7}	50	37
	2×10^{-7}	71	61

nuclease treatment appear not to bind pactamycin at 0 C (fig 8B). On the other hand, 80S ribosomes produced by sodium fluoride treatment of reticulocytes by blocking initiation bind pactamycin readily (fig 8C). For the most part, they are runoff ribosomes and thus free of mRNA and probably other components involved in polypeptide synthesis. These data indicate that ribosomes that bear even a small fragment of mRNA are not able to bind pactamycin at 0 C. This may be due to a direct block of the antibiotic binding site by the mRNA or to a conformational change induced in the ribosome by the mRNA such that this site is inaccessible to the antibiotic. On the other hand, it may instead be due to the presence of some other component(s) of the protein-synthesizing system, present in the polyribosomes, that prevents pactamycin binding.

In a series of experiments using added bacteriophage f2 RNA as mRNA it could be shown convincingly that there is an inverse relationship between the formation of mRNA-bearing

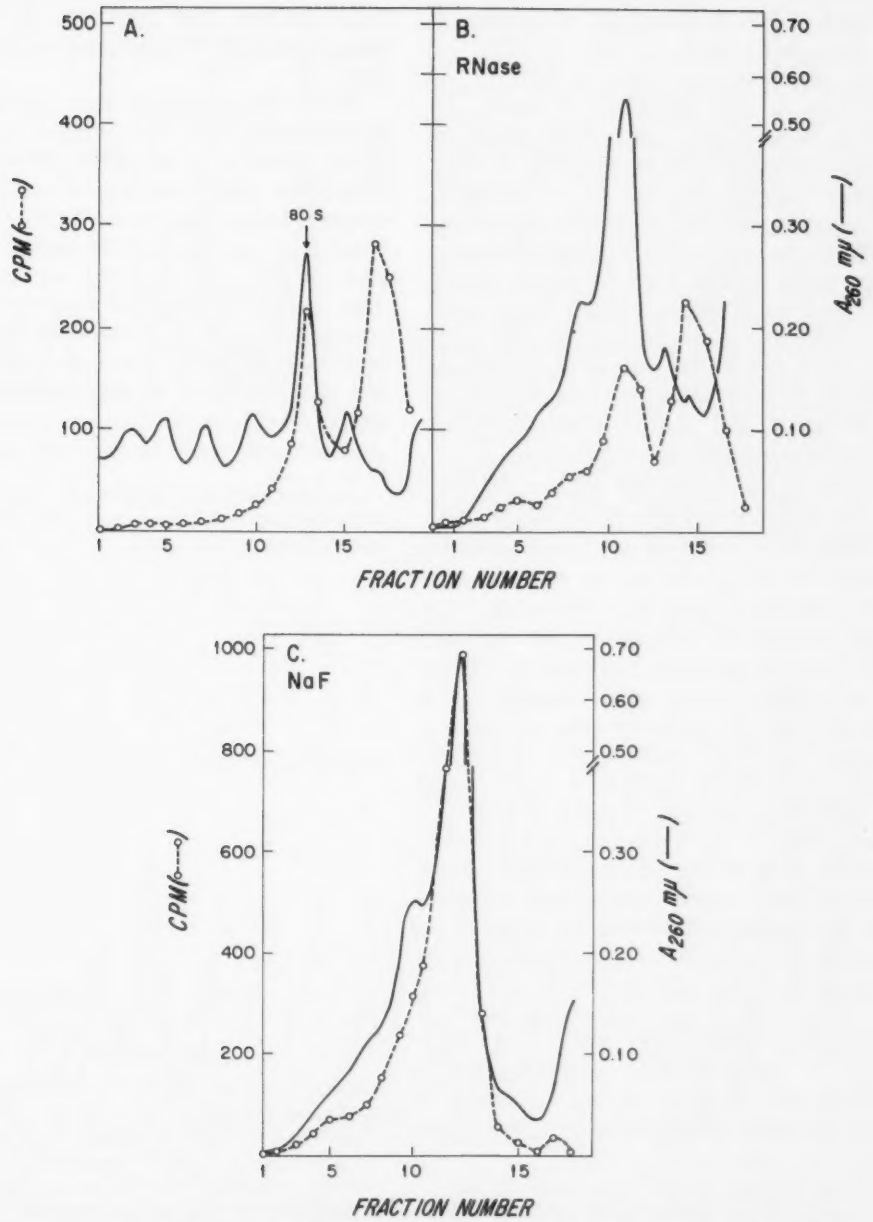

FIGURE 8.—Cold binding of ^{3}H-pactamycin to reticulocyte ribosomes as analyzed by sucrose density gradient centrifugation (from right to left). (*See* ref 24 for experimental details.)

ribosomal complexes engaged in polypeptide synthesis and the cold binding of pactamycin (30). Thus, pactamycin binding decreases as the amount of mRNA added to the reaction is increased (and polypeptide synthesis is thereby increased) and as the binding of mRNA to ribosomes increases with time of incubation. It is not necessary, however, to have active chain elongation taking place on the mRNA-bearing ribosomes in order to block the subsequent cold binding of pactamycin. When elongation and polyribosome formation are prevented by the antibiotics sparsomycin or fusidic acid, mRNA still binds to single ribosomes and pactamycin binding is blocked. From the foregoing one would predict that conditions that lead to the release of ribosomes from mRNA would reinstate their ability to bind pactamycin, and this is what is found when puromycin is added to the reaction.

In contrast to the negligible binding of pactamycin to mRNA-bearing monosomes and polyribosomes at 0 C, adding ^{3}H-pactamycin at 37 C results in binding of the antibiotic to polyribosomes (fig 9). Unlike the rapid binding at 0 C to ribosomes lacking mRNA, binding of pactamycin to polyribosomes is slow, accumulative, and sensitive to inhibitors of elongation such as sparsomycin or chloramphenicol.[7]

These data suggest that pactamycin binds preferentially to free smaller ribosomal subunits, possibly even after initiation complex formation (and possibly to runoff single ribosomes) before monosome formation. The specificity of pactamycin for initiation may thus rest on the fact that the antibiotic binds to free smaller ribosomal subunits or runoff ribosomes, but not to ribosomes (in polysomes) already engaged in chain elongation. The binding of pactamycin to polysomes found at temperatures where protein synthesis takes place would result from the association of a new ini-

[7] Chloramphenicol: NSC-3069; CAS reg. No. 56-75-7; acetamide, 2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl]-.

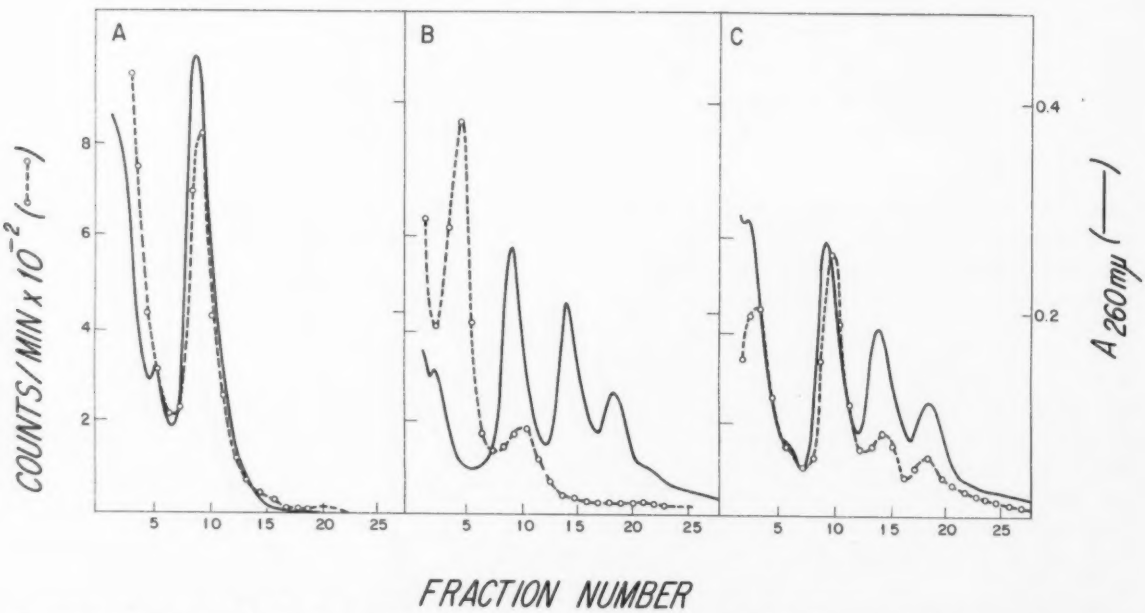

FIGURE 9.—^{3}H-pactamycin binding to f2 RNA-bearing ribosomes at 0 and 35 C. No f2 RNA is present in A and the reaction was always at 0 C. In B and C, f2 RNA-promoted polyribosome formation was allowed to take place (15 min, 35 C). In C, ^{3}H-pactamycin was present throughout the incubation at 35 C, whereas in B, the labeled antibiotic was added at 0 C after completion of incubation. Sucrose density gradient centrifugation was from left to right (30).

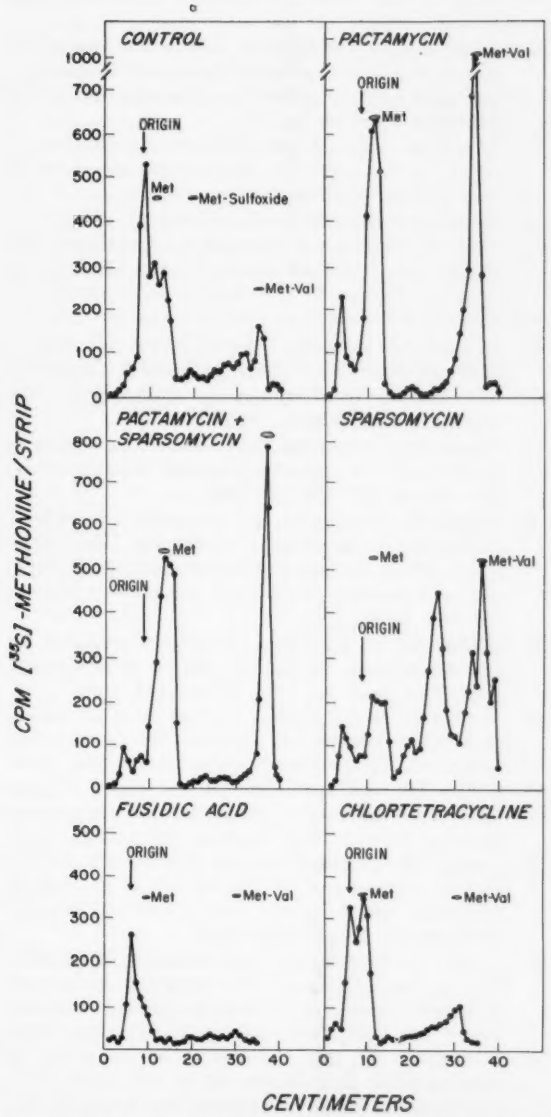

FIGURE 10.—Electrophoretic analysis of the peptides synthesized in the presence of pactamycin and other inhibitors. Reaction mixtures (0.2 ml) were prepared, incubated, and centrifuged through a sucrose gradient as described in ref 31. The inhibitors (pactamycin, 2×10^{-4} M; sparsomycin, 1×10^{-5} M; fusidic acid, 2×10^{-3} M; chlortetracycline, 1×10^{-3} M) when present were added at the beginning. Polysomes were isolated and the pellet was suspended in 100 μl of buffer and incubated with 4 μM of NaOH at 37 C for 15 min. Internal standards: methionine (20 μg), methionine sulfoxide (20 μg), and methionyl-valine (40 μg) were added. An 80-μl sample was applied on Whatman No. 3 MM paper. Counts applied per strip varied from 5000 to 10,000. Electrophoresis was for 2.5 hrs at 3400 volts and 40 mA in the case of A, B, C, and D, and 2 hrs for E and F. The positions of the markers (methionine [met] and methionyl-valine [met-val]) as located with ninhydrin are shown in the figure.

tiation complex bearing pactamycin to the initiation site on the mRNA recently vacated by ribosomal progression down the mRNA. That polypeptide synthesis is required to vacate this site is indicated by the fact that elongation inhibitors decrease the amount of pactamycin that becomes bound to the polysomes. In this way, the step in the protein synthesis sequence blocked by pactamycin need not be peculiar to initiation but may be involved in elongation, such as translocation or acceptor site binding. Thus, only the last ribosome inactivated by bound antibiotic would be unable to move down the mRNA. Such an effect would be viewed grossly (as measured by polysome degradation and globin-chain completion) as an effect on "initiation" even though it is actually "elongation" that is being affected. In order to test such a possibility, the reticulocyte lysate protein-synthesizing system was incubated with initiator ^{35}S-methionyl-tRNA in the presence of pactamycin and the radioactivity remaining attached to the ribosomes was analyzed for its conversion into oligopeptides (31). As shown in figure 10, unlike with sparsomycin where general elongation inhibition results in the appearance of a series of radioactive oligopeptides, with pactamycin there is a selective accumulation of methionyl-valine, the initiator dipeptide. There is, therefore, a block after formation of the first dipeptide.

Using a different system dependent on synthetic mRNA, Cheung et al (32) have also found that dipeptide accumulates in the presence of pactamycin. Since the dipeptide can be released to a considerable extent by puromycin (Kappen LS, unpublished data), it appears that the dipeptidyl-tRNA is in the P-site or in an equivalent site that permits reaction with puromycin. Dipeptide accumulation is found at both 2 and 5 mM Mg^{2+}.

While it appears that in the presence of pactamycin the initiation complex is func-

tional to the extent that it can partake in dipeptide formation, it is defective for two reasons: (*a*) it cannot form tripeptide as noted above, and (*b*) at low magnesium concentrations (2 mM), joining of the larger ribosomal subunit to the smaller initiation complex to form an active, stable, 80S complex is interfered with (33,34). We conclude, therefore, that when pactamycin binds to the smaller ribosomal subunit, it induces a structural alteration in the smaller initiation complex that prevents the formation of a functional 80S complex when the magnesium concentration is low, and it blocks some step after dipeptide formation even at higher magnesium concentrations. At this time, the precise nature of the block in dipeptide elongation is not known.

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Molecular Pharmacology of Plant Lectins: Studies on Ricin and Concanavalin A (NSC-143504)

Arthur P. Grollman,[1] Carl Grunfeld,[1,2] Curtis F. Brewer,[1] and Donald M. Marcus [1,3,4]

SUMMARY

A toxic protein, isolated and purified from extracts of *Ricinus communis*, inhibits protein synthesis in HeLa cells and cell-free lysates prepared from rabbit reticulocytes. The effect on HeLa cells occurs only after a significant delay and can be partially or completely blocked by adding lactose. Low concentrations of ricin cause dissociation of polyribosomes in HeLa cells; this effect parallels inhibition of protein synthesis. Dissociation can be prevented by adding anisomycin, an inhibitor of peptide-bond formation. In separate experiments, binding of α-methyl-D-glucopyranoside (uniformly enriched with 14% ^{13}C) to concanavalin A was studied by pulsed Fourier transform carbon magnetic resonance techniques. The spin-lattice relaxation time for each of the ^{13}C-atoms of the sugar was measured in the absence and presence of zinc and manganese derivatives of concanavalin A, and the contribution of the paramagnetic manganese ion to the relaxation times of the bound saccharide was determined. These data permit calculation of the distances between the ion and the individual carbon atoms and determine the three-dimensional orientation of the sugar relative to the transition metal site in concanavalin A. The results indicate that the sugar binds in the Cl chair conformation with its 3- and 4-carbons closest to the manganese ion at a mean distance of 10 Å. This magnetic resonance method may be used to determine the geometry of complexes formed between various small molecules, including drugs, and macromolecules that contain a binding site for paramagnetic ions.

[Cancer Chemother Rep 58:491–501, 1974]

The term lectin was originally proposed by Boyd (1) to describe a class of proteins that agglutinate erythrocytes and certain other types of cells. This agglutination is inhibited by sugars, a property that has proved useful in determining the specificity of lectins for complex saccharides.

Lectins are most commonly found in the seeds of legumenous plants but also occur in lower vertebrates and in some invertebrates; their biologic properties have been the subject of investigations for more than 80 years (reviewed in ref 2). A recent upsurge of interest

[1] Departments of Pharmacology, Microbiology and Immunology, Medicine, and the Division of Biological Sciences, Department of Molecular Biology, Albert Einstein College of Medicine, New York, NY.

[2] Medical Scientist Trainee of the US Public Health Service.

[3] Career Scientist of the Health Research Council, New York, NY.

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in these proteins has resulted from their demonstrated ability to preferentially agglutinate tumor cells (3,4) and cells transformed by chemicals and viruses (5,6). Several lectins, including ricin (7) and concanavalin A (Con A) (8,9), suppress the growth of tumors in experimental animals; purified preparations of ricin have been successfully used in the treatment of human cervical cancer (10).

The biochemical mechanisms by which lectins interact with receptors on cell surfaces and exert their toxic effects are of particular interest to pharmacology. In the present paper, we will examine these questions with reference to ricin and Con A. Ricin, isolated from the castor bean *Ricinus communis*, is the most toxic of the known lectins (2,11). To elucidate the mechanism of its toxic effect, we have studied the action of this lectin on macromolecular synthesis in HeLa cells.

The primary structure of ricin is not known; however, the protein chemistry of Con A, a protein of the jack bean *Canavalia ensiformis*, has been extensively studied (cf 2, 12–14). The second part of our paper describes the interaction of Con A with saccharides as studied by magnetic resonance techniques; the final section deals with interactive computer graphics as a means of studying binding sites in Con A and other proteins.

STUDIES ON RICIN

Stillmark first reported (15) that a crude proteinaceous extract of *Ricinus communis* was toxic to mice and agglutinated erythrocytes. Soon afterwards, Ehrlich (cf 16) used similar extracts prepared from *Ricinus communis* to elucidate various properties of immunologic systems. Among other fundamental observations, he established immunity to a lethal dose of ricin by previously administering small amounts of this lectin. Ehrlich found that serum obtained from ricin-treated animals was capable of specifically neutralizing the toxin. He also demonstrated (17) severe inflammatory and necrotic changes in rats which occur following injection of sublethal concentrations of ricin (11,18).

Purification and Properties

The structure and toxic function of proteins isolated from *Ricinus communis* have been reviewed by Funatsu (11) and Olsnes (19). The major toxic and agglutinating activities of the crude extract can be separated. The molecular weight of the toxic protein is approximately 60,000; it consists of two subunits that are readily dissociated by treatment with β-mercaptoethanol (11). This treatment markedly reduces toxicity towards mice (20). The subunits are clearly different, but the precise amino acid composition and identity of the *N*- and *C*-terminal amino acids are disputed (11,21,22).

Several methods have been described for preparing the toxic protein ricin (11,20–24); earlier preparations (25), although crystalline, appear to contain several components.[5] There are differences in the properties of purified ricin reported by various laboratories; the apparent discrepancies may result from studying different forms of the protein which are found at various stages of growth of *Ricinus communis* seeds (cf 11).

The mechanism by which ricin exerts its toxic effect on animal cells has not been clearly established. Lin et al (26) demonstrated the cytotoxic action of the protein against Ehrlich ascites cells and showed that protein and DNA synthesis in these cells were inhibited by the lectin while RNA synthesis was unaffected. Subsequently, these investigators (27) described disaggregation of liver polyribosomes in ricin-treated rats and attributed these effects to an observed increase in the activity of ribonuclease. Olsnes and Pihl arrived at a different conclusion based on studying the effects of ricin on reticulocyte lysates. These investigators reported that nascent peptide remained bound to polyribosomes in the presence of ricin, and they concluded that the lectin inhibited elongation of the peptide chain (28). Experiments in our laboratory, suggesting that other mechanisms may be involved, are presented below.

Effects on Protein Synthesis in HeLa Cells

We have isolated and purified a toxic protein (ricin) from extracts of *Ricinus com-*

[5] Grunfeld C, and Grollman AP. Manuscript in preparation.

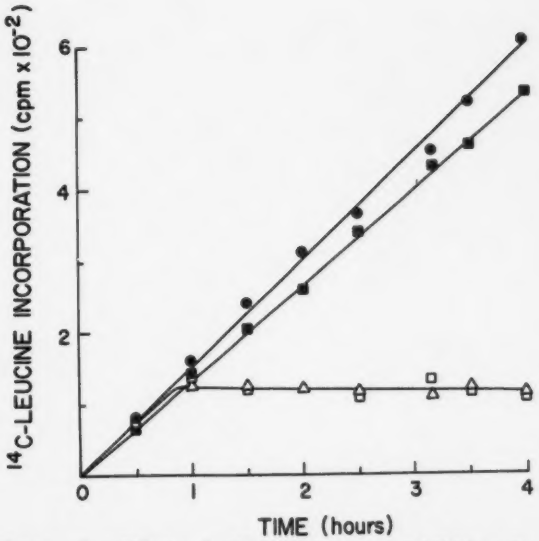

FIGURE 1.—Effect of disaccharides on inhibition by ricin of leucine incorporation in HeLa cells. HeLa cells (4×10^5 ml) were incubated at 37°C in leucine-depleted Eagle's medium containing 5% fetal calf serum and (^{14}C)-leucine. Protein synthesis was measured as described elsewhere (29). Ricin, final concentration 1 μg/ml, was mixed with disaccharides (10 mM) for 5 min and the mixture was added to the cells at 0 time. Aliquots were removed and 10 volumes of cold saline solution were added. Cells were collected by centrifugation, lysed in water at room temperature, and trichloroacetic acid was added to a final concentration of 10%. The acid-insoluble precipitate was collected on glass fiber filters and the radioactivity was determined (29). ●——● = Uninhibited control; ▲——▲ = ricin and cellobiose; ■——■ = ricin and lactose; □——□ = ricin.

munis.[5] This protein inhibits incorporation of radioactive amino acids into protein in suspension cultures of HeLa cells. This effect starts 1 hour after 1μg/ml of ricin is added to cells (fig 1). Inhibition of protein synthesis is further delayed but not completely blocked if galactose or galactose-containing saccharides are present in the medium. Lactose, *O*-β-D-galactopyranosyl-(1→4)-D glucose (10 mM) prevents the inhibitory effects of 1 μg/ml of ricin while similar concentrations of cellobiose, *O*-β-D-glucopyranosyl-(1→4)-D glucose are ineffective (fig 1).

Low concentrations of ricin induce dissociation of polyribosomes; this effect parallels inhibition of protein synthesis (fig 2). One hour after the addition of ricin (1 μg/ml), the majority of polyribosomes are dissociated into trimers, dimers, and monomers; after 3 hours, the total number of ribosomes is decreased and only single ribosomes are present. Breakdown of polyribosomes induced by ricin can be prevented by simultaneously adding anisomycin (30), an inhibitor of peptide-bond formation, indicating that protein synthesis is required for this effect to occur.

Effects on Protein Synthesis in Reticulocyte Lysates

Inhibition of protein synthesis by ricin can also be demonstrated in reticulocyte lysates (fig 3). At low concentrations, the onset of this effect is delayed for several minutes. Inhibition of protein synthesis in cell-free extracts is not blocked by addition of lactose.

Mechanism of Action

Our experiments were designed to determine the biochemical mechanism responsible for the cytotoxic action of ricin on animal cells. The observed effects on DNA synthesis in HeLa cells are probably secondary to a primary effect on protein synthesis (cf 29) since the latter process is required for the concurrent synthesis of DNA in animal cells (31, 32). This conclusion is supported by the strong inhibitory effects of ricin on protein synthesis in reticulocyte lysates, conditions under which DNA is not formed and globin is the only macromolecule synthesized. All known primary inhibitors of protein synthesis in eukaryotes also inhibit DNA synthesis (cf 33).

Other investigators have attributed the inhibitory effects of ricin on protein synthesis to increased activity of ribonuclease (27) or, alternatively, to selective inhibition of peptide chain elongation (28). In our experiments, ricin appears to inhibit initiation of peptide chains; several pieces of evidence support this conclusion. In ricin-treated HeLa cells, dissociation of the larger polyribosomes parallels inhibition of protein synthesis. Inhibitors of chain elongation (such as anisomycin) block dissociation, suggesting that ribonuclease is

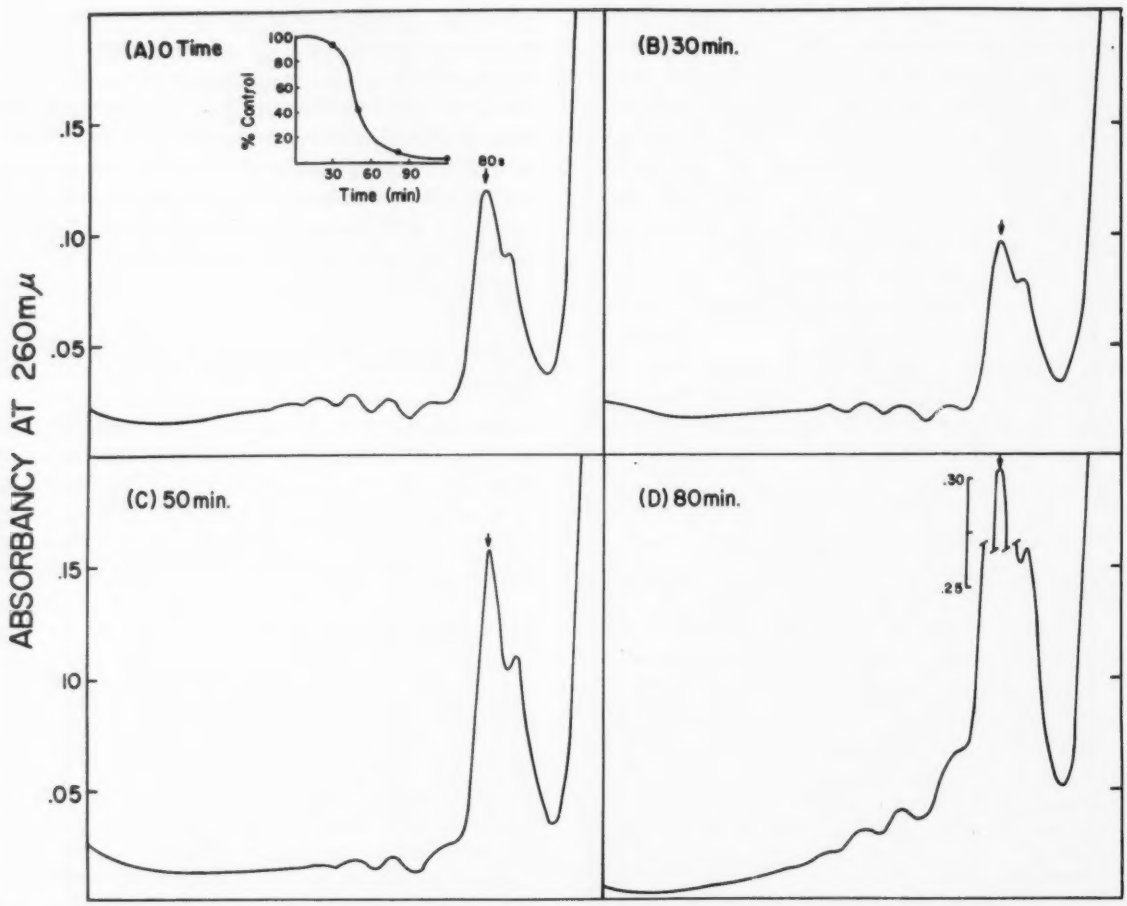

FIGURE 2.—Effects of ricin on polyribosomes in HeLa cells. HeLa cells (4×10^5/ml) were incubated at 37 C in Eagle's medium. Ricin was added, at a final concentration of 1 μg/ml, and the incubation continued for the indicated periods of time. The cells were lysed, nuclei were removed by centrifugation, and the supernatant solution containing polyribosomes was layered onto 15%–30% (w/v) sucrose density gradients as described previously (29). Samples were centrifuged for 2.5 hrs at 25,000 rpm in a Spinco SW 27 rotor. Absorbance at 260 mμ was monitored by means of a flow cell attached to a Gilford spectrophotometer. Sedimentation is from right to left. The insert records the degree of inhibition of protein synthesis at various times during the experiment as measured in aliquots of the cell suspension by determining the incorporation of (^{14}C)-leucine into trichloroacetic acid-insoluble material.

not involved in the effect.[6] In the cell-free reticulocyte lysate, ricin inhibits protein synthesis after a delay of several minutes—the period of time required to complete chains of globin already bound to the polyribosome. These kinetics are characteristic of agents that affect initiation; inhibitors of chain elongation block protein synthesis without measurable delay (33). However, breakdown of polyribosomes that usually accompanies inhibition of initiation is not observed in lysates (28). These results suggest that alternative explanations of the observed delay should be considered.

Interactions With Cell Surfaces

Inhibition of protein synthesis in ricin-treated HeLa cells is observed only after a sig-

[6] *See* footnote 5.

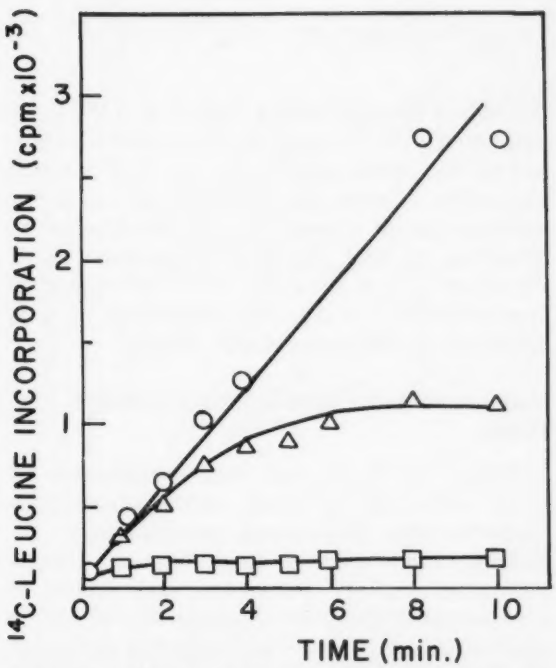

FIGURE 3.—Effect of ricin on incorporation of leucine into a reticulocyte lysate. Rabbit reticulocyte lysates were prepared according to previously published procedures (29). The lysate was incubated at 33°C in the presence of hemin; Borsook amino acids; 10 mM tris HCl (pH 7.4); 75 mM KCl; 1 mM ATP; 0.02 mM ATP; 15 mM creatine phosphate; 0.9 mg/ml creatine phosphokinase; 2 mM $MgCl_2$; 6 mM 2-mercaptoethanol; and 2 μM (^{14}C)-leucine (315 curies/mole). Aliquots were removed and added to a cold solution of 10% trichloroacetic acid containing (^{12}C)-leucine. Samples were heated for 15 min at 95°C and then chilled in an ice bath. Precipitates were collected and the radioactivity determined as described in the legend to fig 1. ○——○ = Uninhibited control; △——△ = ricin, 1.7 μg/ml; □——□ = ricin, 17 μg/ml.

nificant delay. The extent of delay depends on the concentration of ricin. The effects of ricin can be blocked, at least partially, if lactose or other saccharides are added within 5 minutes after adding the lectin. In contrast, antibiotics and other small molecules that inhibit protein synthesis in HeLa cells exert their full inhibitory effects during this period of time (33). Rapid inhibition of protein synthesis in cell-free lysates by ricin suggests that intracellular metabolism or activation of ricin is probably not a significant factor in the observed delay. Binding or subsequent penetration seem more likely to be rate-limiting in this process.

Ricin-induced agglutination of erythrocytes is blocked by galactose and galactose-containing oligosaccharides and polysaccharides (24,34,35). Agglutination of HeLa cells by ricin can also be prevented by adding saccharides of similar specificity.[6] The intracellular effects of ricin on protein synthesis can be selectively blocked by the same saccharides that prevent attachment of ricin to the cell surface. Olsnes and Pihl (20) have shown that the smaller of the two peptide chains of ricin is responsible for inhibition of protein synthesis in cell-free lysates. The isolated subunits are inactive against intact cells; the data suggest that the larger peptide chain is responsible for galactose-specific binding to the cell membrane.

BINDING OF SACCHARIDES TO CON A

Con A is composed of identical subunits with a molecular weight of 27,000. Between pH 3.5 and 5.6 the lectin exists as a dimer; at higher pH tetramers are formed (36,37). Each monomeric unit has one site that binds transition metal ions and another that binds calcium ions; both sites must be occupied for saccharide binding to occur (38,39). Derivatives in which Zn^{2+}, Co^{2+}, or Mn^{2+} ions occupy the transition metal site bind saccharides with equal affinity.

The affinity of lectins for specific carbohydrates afforded an opportunity to study the three-dimensional structure of small molecule-macromolecule complexes in solution. We have used magnetic resonance techniques, measuring the relaxation times of the carbon nuclei of free and bound saccharides, to accomplish this objective. Con A provided a convenient source of purified, relatively nontoxic material for these experiments; the crystal structure and primary amino acid sequence of this lectin have been established (9–11).

Nuclear Magnetic Resonance Studies

The principle of our experimental approach is as follows: Con A is a metalloprotein with separate binding sites for calcium and transition metal ions; the requirement for transition metal ion is equally satisfied by zinc or manganese. Thus, Con A can be prepared in a paramagnetic (manganese) or diamagnetic (zinc) form, both of which have similar binding affinities for saccharides. Paramagnetic ions decrease the relaxation times of nuclei in their environment; this effect is inversely related (by the sixth power) to the distance between the ion and the nucleus in question (40). Thus, by measuring the contribution of the paramagnetic ion to the relaxation time of each carbon atom of a saccharide that binds Con A, the position and orientation of the bound molecule relative to the ion can be determined.

Theoretical Aspects

Several equations are required for the calculations used in these experiments; the essential ones are given below, and further details of the relevant relaxation theory can be found elsewhere (40,41).

$$T_1^{-1} = T_{1o}^{-1} + f\,(T_{1M} + \tau_m)^{-1} \qquad (1)$$

where T_1 and T_{1o} are the spin-lattice relaxation times of the sugar carbons in the presence and absence of protein respectively; f is the ratio of bound to unbound sugar in solution; T_{1M} is the spin-lattice relaxation time of the carbons of the sugar bound to the protein; and τ_m is the residence time of the sugar bound to the protein.

The paramagnetic contribution, T_{1P}, is determined by measuring the difference between the zinc and manganese forms of the protein. Thus,

$$T_{1P}^{-1} = (T_{1M})^{-1}_{\text{Mn - Con A}} - (T_{1M})^{-1}_{\text{Zn - Con A}} \qquad (2)$$

which assumes that the $^{13}C - {}^{1}H$ diamagnetic-dipolar relaxation contribution to T_{1M} for the sugar carbons bound to manganese-Con A is the same as that when bound to zinc-Con A. T_{1P} is related to the intranuclear distance by:

$$T_{1P}^{-1} = (^2/_5)g^2\beta^2\gamma_I^2 S(S+1) r_{CM}^{-6} \tau_c (1 + \omega_I^2 \tau_c^2)^{-1} \qquad (3)$$

where g is the electronic g factor; B is the Bohr magneton; S is the electron spin quantum number of the paramagnetic ion; r_{CM} is the metal ion-carbon intranuclear distance; and τ_c is the electron-nuclear correlation time for dipolar interactions at 23.4 KG. For manganese-Con A, the values of $g = 2.0$ and $S = 5/2$ provide good approximation (cf 41). Determination of τ_c is described in detail elsewhere (41,42).

Measurements of Spin-Lattice Relaxation Times

Values for T_1 of each carbon resonance of (^{13}C) uniformly enriched α-methyl-D-glucopyranoside were determined simultaneously by plotting the observed magnetization (longitudinal relaxation) recovery of each carbon as a function of the pulse interval (fig 4). T_1 is approximately 1 second for each ring carbon of the free sugar and approximately half this value for carbon 6. T_1 is reduced for all (^{13}C) carbon atoms when the sugar binds to zinc-Con A and decreases even further when bound to manganese-Con A due to the presence of the paramagnetic ion (fig 4).

Three-Dimensional Geometry of the Complex

Using equation (3) and experimentally determined values for T_{1P}, the distances between the manganese ion and each carbon of the bound sugar were calculated (table 1). The mean distance separating the sugar from the manganese ion is about 10 Å. The nonreducing end of the sugar is closest to the manganese ion (cf fig 5) and is, therefore, oriented towards the center of the protein. No changes in chemical shifts were observed in the presence of Con A; we therefore assume that the bound sugar retains the Cl chair conformation that exists in solution.

A computer program was used to search for orientations and distances between the manganese ion and the carbon atoms of the sugar such that the ratio of the distances generated

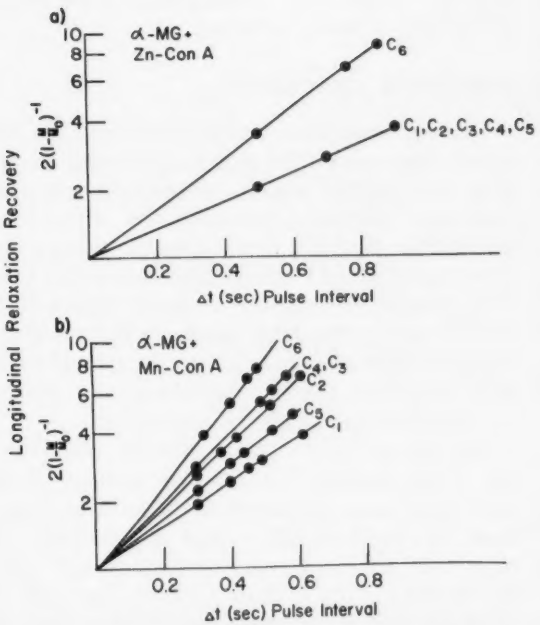

FIGURE 4.—^{13}C magnetization recovery plotted as a function of pulse interval. $\Delta t = T_{1M}$ when $2(1-M/M_o)^{-1} = 2.718$. For experimental details, *see* ref 41.

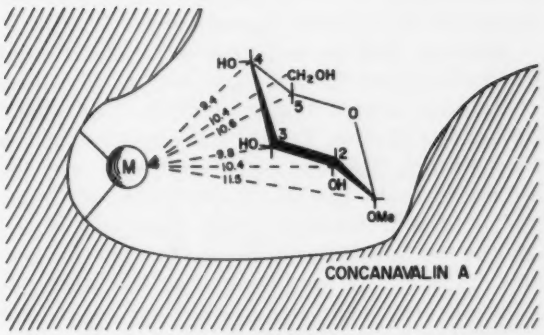

FIGURE 5.—Orientation and position of α-methyl-β-D-glucopyranoside relative to the transition metal ion site in Con A as calculated from carbon magnetic resonance data. Distances shown are given in Å.

correspond to the best fit of the ratio of the experimental T_{1P} data. Coordinates of the α-anomer in the Cl chair conformation, determined by X-ray diffraction techniques (43), were used for the internal bond angles and distances. This method for determining manganese-carbon distances for the sugar does not require an experimental value for τ_c. The unique orientation and position found for the sugar were in good agreement with similar data calculated using equation (3) and experimentally determined value (42) for τ_c. The computer-calculated and experimental values differ substantially if the average distance from the sugar to the manganese ion is more than 10.5 or less than 9.5 Å.

Comparison Between Con A-Saccharide Complexes in Solution and in the Crystalline State

At the same time that our initial results were reported (44), two groups of crystallographers (12,13) independently completed X-ray diffraction studies of Con A-saccharide complexes. They concluded that the binding of carbohydrates occurs 20 Å from the transition metal ion site; in contrast, our magnetic resonance data (41,44) indicate that this site was only 10 Å from the metal. Addi-

TABLE 1.—Experimental and computer-fitted distances between manganese ion and the carbon atoms of α-methyl-β-D-glucopyranoside*

Carbon	T_{1P} (sec)	Distances (Å)	
		Experimental	Computer-fitted
1	0.091	11.7	11.5
2	0.040	10.2	10.4
3	0.028	9.6	9.8
4	0.028	9.6	9.4
5	0.057	10.8	10.6
6	0.038	10.1	10.4

*For experimental details, *see* ref 41.

tional studies have been reported that may account for the apparent discrepancy.

Edelman and his collaborators (12,45) used *o*-iodophenyl-β-D-glucopyranoside, a glycoside containing an aromatic moiety, for their diffraction studies. Based on the location of the iodine atom, the position of the sugar was inferred to be in the major cleft with the nonreducing end directed away from the manganese ion and towards the surface of the protein (12). It is pertinent that the glucopyranoside ring was not directly observed in this diffraction study and that crystals of native Con A are cracked and dissolved in the presence of α-methyl-D-mannopyranoside (46).

Hardman and Ainsworth, who reported formation of a crystalline myo-inositol-Con A complex (13), have apparently been unable to repeat these original experiments (46); they suggest that methyl-*p*-hydroxybenzoate, added to suppress bacterial growth during crystallization, may account for the increased electron densities attributed in their earlier studies to myo-inositol. These investigators have further shown that many nonpolar compounds, including methyl-*p*-hydroxybenzoate, phenylphosphate, *o*-iodobenzoic acid, and dimethylmercury, form crystalline complexes with Con A in which these molecules are bound 20 Å from the metal (46). Of particular importance was their demonstration that *o*-iodophenyl-β-D-galactopyranoside binds in a similar manner to the nonpolar site. This saccharide does not bind to Con A in solution and it would appear that the site at 20 Å, previously identified (12,13,45) as the general "carbohydrate binding site," binds, instead, a variety of relatively nonpolar molecules (46).

The saccharide used in the studies of Edelman et al (*o*-iodophenyl-β-D-glucopyranoside) may also bind the nonpolar site. We have repeated our nuclear magnetic resonance studies with this molecule, enriched in (^{13}C), and have shown that only a fraction of the bound material is specifically displaced by α-methyl-β-D-mannopyranoside. Most of the bound saccharide demonstrates only a diamagnetic contribution to T_1 (41) and is, thus, located more than 18 Å from the metal. These experiments provide direct demonstration of at least two binding sites for *o*-iodophenyl-β-D-glucopyranoside in aqueous solution.

COMPUTER GRAPHICS

Protein chemists, crystallographers, and others who work with molecular models of proteins frequently encounter certain practical problems. Accurate models are difficult to build from electron density maps, components are expensive, and coordinates calculated from such models are subject to error. Space-filling models are even less useful, since positions of atoms in proteins cannot be accurately measured and the models are physically difficult to support in the proper conformation.

The use of interactive computer graphics in the representation of complex biologic molecules has been reviewed by Katz and Levinthal (47) and by Barry and North (48). We have used this technique to display and study the binding sites of Con A. Levinthal and coworkers, the first group to pioneer this approach, developed the concept of a centralized time-sharing computer with a three-dimensional display which could be used in an interactive manner to display and manipulate macromolecules (49).

The computer system used for our experiments was developed by Feldmann et al and has been described elsewhere (50). It consists of an Adage AGT/30 display linked with a time-sharing DEC PDP-10 computer. Programs require 12,000–55,000 words of the 36-bit word PDP-10 computer, depending on the size of the molecule displayed. At present, molecules containing up to 4000 atoms can be displayed and manipulated. Displays are viewed with stereoglasses or by rotating the two-dimensional image to give a three-dimensional effect. The user interacts with the system and manipulates the display with a series of manual controls; the system is arranged to print out data, store spectral information, or take photographs and movies of the display screen. A routine is available that links the display image to the ORTEP (51) or PLUTO plotting programs; the latter was used to construct the stereogram of Con A shown in figure 6.

As presently constituted, the computer can perform the following operations: (*a*) view

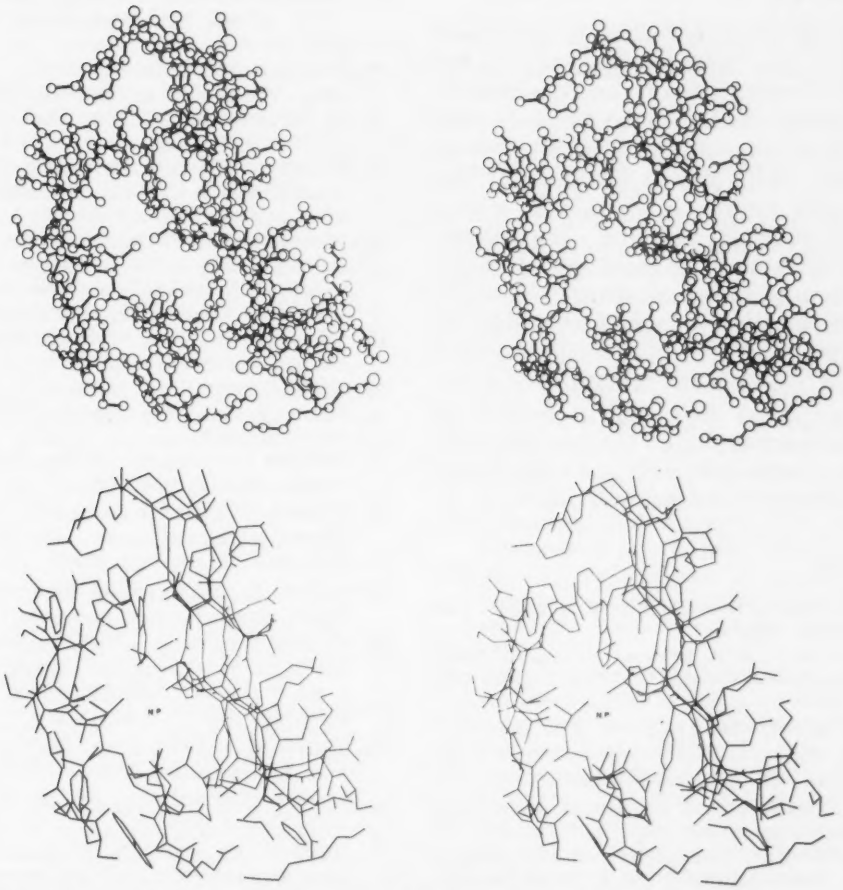

FIGURE 6.—Stereogram of the nonpolar binding region of Con A. Binding appears to involve π stacking of nonpolar compounds with the aromatic side chain of phenylalanine-192 (46). This figure was generated by a computer graphics display developed by Feldmann et al (50) utilizing atomic coordinates for Con A provided by Dr. Karl Hardman.

or rotate part or all of the molecule, (*b*) compute distances between any given atoms, (*c*) display space-filling aspects, (*d*) determine van der Waal's interactions, (*e*) alter conformations, (*f*) utilize crystallographic data on all small molecules and some 20 proteins reported in the literature over the past decade, (*g*) simultaneously display small molecules, inhibitors, or substrates, and (*h*) number or letter various atoms or groups in macromolecules (47,50).

CONCLUSIONS

We conclude from our studies with ricin that this lectin is a primary inhibitor of protein synthesis—an effect that may account for many of its toxic actions on animal cells. The delay that precedes onset of inhibition of protein synthesis in HeLa cells may be related to the time required for ricin to bind and penetrate the cell membrane. The effects of ricin on intact cells are blocked or delayed by galactose-containing saccharides, a phenomenon that

also appears related to the initial binding process.

Magnetic resonance experiments were used to determine the relative position of the carbohydrate-binding site of Con A. α-Methyl-D-glucopyranoside binds in the Cl chair conformation at a mean distance of 10 Å from the transition metal site in the protein. The techniques used for this study provide a general approach for studying the three-dimensional orientation of small organic molecules bound to proteins in solution and can be applied to a variety of small molecules, including drugs, that bind macromolecules containing paramagnetic ions. The accuracy of this method rivals X-ray diffraction techniques in that precise assignments of binding geometry can be made; most importantly, the experiments can be performed in aqueous solution.

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Oncolytic Action of Prostaglandins [1,2]

Herbert I. Jacobson [3]

The work I will describe is in progress. We are pursuing a lead that was uncovered during a study of the process by which prostaglandins terminate the luteal state in rabbits.

The corpus luteum of the rabbit is an estrogen-dependent organ. After the process of ovulation and luteinization of the mature ovarian follicle has been initiated by its exposure to pituitary luteinizing hormone, the necessary and sufficient hormonal requirement for maintenance of luteal structure and function is estrogen (1,2).

If the ova released are not fertilized, the corpora lutea persist for about 18 days, during which time the rabbit is said to be pseudopregnant. The corpus luteum of the pseudopregnant rabbit, like all other estrogen-dependent tissues, contains a cytoplasmic estrogen receptor protein (3).

We have examined corpora lutea obtained from rabbits at different intervals following induction of the pseudopregnant state by sterile mating. As shown by the data plotted in figure 1, the mean luteal weight increases steadily for 16 days, then falls abruptly, and is quite small by the 18th day when corpora are regressing and the return to the estrous state is imminent. These last few days of pseudopregnancy also constitute the time interval during which luteal production of progesterone declines.

Figure 1 also illustrates the concentration of cytoplasmic estrogen receptor in the uteri and in the corpora lutea of these rabbits, expressed in femtomoles (fM)/mg wet weight of tissue. As the pseudopregnant state terminates, the uterine content of estrogen receptor rises sharply from the low value to which it has been suppressed at the initiation of pseudopregnancy (3). We have established that this response is in fact a suppression of uterine estrogen receptor by progesterone, which is relieved as luteal production of that hormone dwindles (4).

In contrast to these late events that signal imminent termination of the pseudopregnant state, the concentration of estrogen receptor in luteal tissue reaches its peak and begins to decline a full week earlier, in mid-pseudopregnancy (3).

This suggests that in the rabbit, a possible chain of events by which the luteal state is terminated includes the following elements:

1. Young luteal cells contain cytoplasmic

LUTEAL AND UTERINE CONTENT OF AVAILABLE CYTOPLASMIC ESTROGEN RECEPTOR AND LUTEAL WEIGHT IN PSEUDOPREGNANT NEW ZEALAND RABBITS.

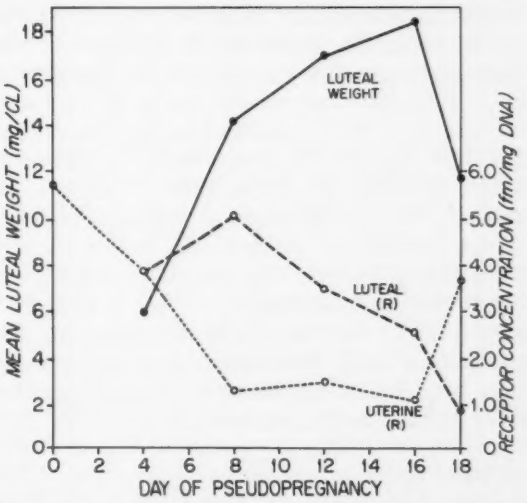

FIGURE 1.—Cytoplasmic estrogen-receptor concentration in luteal tissue and uterus and mean weight of corpora lutea at different stages of pseudopregnancy in New Zealand rabbits. (Plotted from the data in ref 3.)

[1] Supported by the Ford Foundation for the work in reproduction physiology and by the Damon Runyon Memorial Fund for the ongoing tumor studies.

[2] Prostaglandins used in these experiments were supplied by the Upjohn Company, Kalamazoo, Mich.

[3] Department of Obstetrics and Gynecology, Albany Medical College of Union University, NY.

estrogen receptor in amounts far in excess of the quantity required for reaction with the number of estrogen molecules that enter their cytoplasmic compartments.

2. As the luteal state progresses, a process of unknown nature alternates luteal cell estrogen-receptor content, perhaps by slowing synthesis, by accelerating degradation, or both.
3. The decline in receptor concentration doesn't affect cell function until the amount remaining is inadequate to combine with all of the estrogen molecules that enter the luteal cells.
4. Cell function is then impaired due to an inadequate content of receptor-estrogen complex. The estrogen-dependent cells have been rendered incapable of "perceiving" estrogen and deteriorate as if suffering estrogen deprivation despite the presence of an adequate hormone level.

The luteolytic process responsible for the termination of pseudopregnancy in rabbits occurs in response to rising levels in the plasma of the agent prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (5), produced by the uterine endometrium (6). Hysterectomy will prolong the pseudopregnant state, while the injection of $PGF_{2\alpha}$ will end it prematurely. One day after the subcutaneous (sc) administration of 400 μg/kg of $PGF_{2\alpha}$ to rabbits that are 9 days' pseudopregnant we find that the plasma progesterone content has fallen sharply, the mean luteal weight is depressed, the concentration of estrogen receptor in the uterus has doubled, and in the corpora lutea it has halved. The entire second half of the 18-day pseudopregnant period is thus telescoped into 1 day insofar as are concerned the physiologic and biochemical parameters we have been following. However, if our hypothesis is to have merit, we would have to find that injection of $PGF_{2\alpha}$ provokes a fall in luteal estrogen-receptor content *before* the other changes take place. In figure 2 are the data showing this to be the case. The entire timespan on this graph is the 24-hour period following $PGF_{2\alpha}$ treatment of 9-day pseudopregnant rabbits. A decline in luteal weight and

CONCENTRATION OF ESTROGEN RECEPTOR IN UTERI AND CORPORA LUTEA OF RABBITS FOLLOWING TERMINATION OF PSEUDOPREGNANCY ON DAY 9 BY $PGF_{2\alpha}$ ADMINISTRATION

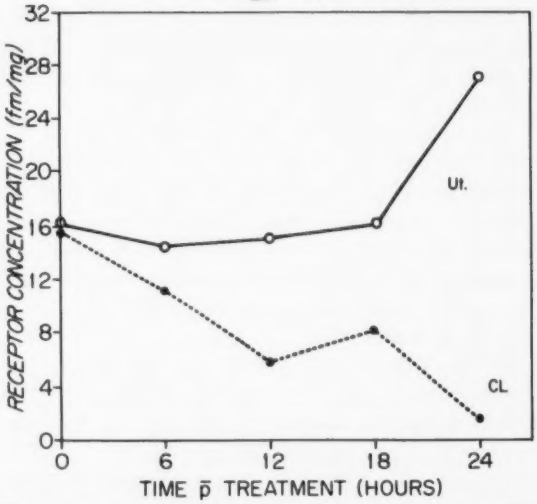

FIGURE 2.—Cytoplasmic estrogen-receptor concentration in corpora lutea and uteri of Dutch-belted rabbits at 6-hr intervals following administration of $PGF_{2\alpha}$ (400 μg/kg) sc on Day 9 of pseudopregnancy.

in plasma progesterone content and an increase in uterine content of estrogen receptor were all undetectable until at least 18 hours after treatment. However, luteal receptor content was measurably diminished by 6 hours after prostaglandin injection (7).

The proposition that $PGF_{2\alpha}$ acts directly rather than systemically on the estrogen receptor of corpus luteum would be firmly supported if this same action could be demonstrated in vitro. In order to determine whether explants of luteal tissue would lose their estrogen receptor when incubated with a culture medium containing $PGF_{2\alpha}$, it was first necessary to devise a culture system in which the receptor would survive without the prostaglandin. For these experiments, the ovaries of 9-day pseudopregnant rabbits were removed under Nembutal anesthesia. The corpora were dissected free of interstitial tissue and were cut into quarters. These were distributed among four culture flasks, so that each rabbit

generated four nearly identical tissue samples. Each rabbit thus provided the material for a four-point kinetic curve.

The incubations were carried out in 2 ml of tissue culture medium 199 at 4 C under an atmosphere of 95%/5% CO_2-oxygen. Reactions were stopped by dropping the bits of tissue into liquid nitrogen, in which they were preserved until analyzed.

The graphs in figure 3 describe the survival of cytoplasmic estrogen receptor in rabbit luteal explants incubated in the presence and in the absence of 10^{-7} M $PGF_{2\alpha}$. Inclusion of prostaglandin in the culture medium causes substantial loss of receptor in 2 hours, while only slight decay is seen in control cultures.

Table 1 summarizes the data obtained in several such studies. The mean decay of receptor in 2 hours was 5.5 fM/mg in control cultures and 16.3 fM/mg in prostaglandin-containing cultures. The difference is large and significant ($P < 0.05$).

The formula for $PGF_{2\alpha}$ is shown in figure 4. With it is the formula for the prostaglandin analog 7-oxa-13-prostynoic acid (OPA) that we obtained from Dr. Josef Fried at the University of Chicago. This material has been shown to be an inhibitor for the action of prostaglandin in several mammalian systems (8).

In similar culture experiments that require repetition and confirmation (work in progress) we have observed that there is saturable binding of tritium-labeled prostaglandin to rabbit luteal tissue, that inclusion of the inhibitor OPA in the culture medium will reduce the uptake of tritium by the tissue, and that under similar conditions such inclusion will reduce the $PGF_{2\alpha}$-provoked decline in luteal content of estrogen receptor.

The conclusions we derive from these findings (some firm and some rather tentative) are as follows: $PGF_{2\alpha}$ elaborated by the pseudopregnant rabbit has a direct effect on the biology of the corpus luteum, an action

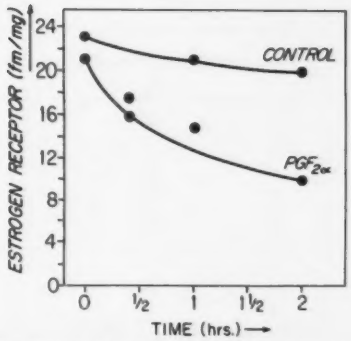

FIGURE 3.—Survival of cytoplasmic estrogen receptor in luteal tissue obtained from 9-day pseudopregnant Dutch-belted rabbits, incubated up to 2 hrs in medium 199 at 4 C in the presence and absence of 10^{-7} M $PGF_{2\alpha}$.

TABLE 1.—Effect of 10^{-7} M $PGF_{2\alpha}$ on survival of cytoplasmic estrogen receptor in luteal explants incubated for 2 hours*

	Receptor concentration (fM/mg wet wt of tissue)†			
	t = 0	t = 2 hrs	2-hr decline	
No $PGF_{2\alpha}$	22.8	19.5	3.3	
	21.7	17.7	4.0	
	22.8	13.8	9.0	
		Mean:	5.5	
10^{-7}M $PGF_{2\alpha}$	22.4	9.1	13.3	
	20.0	4.6	17.8	
	22.4	4.5	17.9	
		Mean:	16.3	($P < 0.05$)

*Culture conditions: tissue culture medium 199, 95%/5% CO_2-O_2, 4 C.
†t = time.

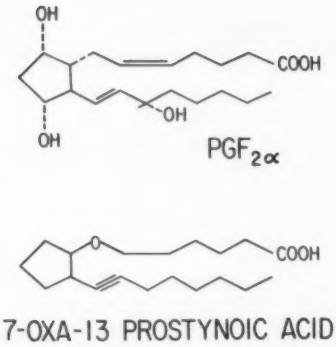

FIGURE 4.—Structural formulas of $PGF_{2\alpha}$ and the analog 7-oxa-13-prostynoic acid.

possibly mediated through binding to prostaglandin receptor sites. An early effect of this encounter, which is manifest before we detect any overt signs of change in luteal structure or function, is the decline in tissue content of cytoplasmic estrogen receptor, this in turn rendering these estrogen-dependent cells incapable of responding to estrogen, leading to their demise. We then asked ourselves whether this action of $PGF_{2\alpha}$ was unique to rabbit luteal tissue, or whether we might also see $PGF_{2\alpha}$ impairment of response to estrogen in other tissues that contain a cytoplasmic estrogen receptor.

Table 2 contains a partial list of candidates for consideration. These are tissues that respond to estrogen in the manner indicated in the right-hand column. The question is whether any of these organ responses can in fact be impaired through depletion of their content of estrogen receptor brought about by administration of prostaglandin. Obviously the most attractive target for study is the estrogen-dependent mammary cancer because of the ease with which its response to estrogen can be measured in rats. It is a nondestructive test, and the only laboratory implements required are a caliper and ruler for determining the dimensions of palpable tumors.

We therefore began the next series of experiments with a *new idea* in mind:

Some tumors are hormone dependent.

Can we devise a treatment that will deplete a tumor of the receptor for the hormone on which it is dependent?

Can we, in this manner, deprive a hormone-dependent tumor of support by its hormone?

Can we thereby produce tumor regression?

TABLE 2.—Some mammalian tissues that are responsive to estrogen and that contain a cytoplasmic estrogen receptor

Tissue	Response to estrogen
Endometrium	Proliferation
Vagina	Tissue "maturation"
Hypothalamus	Inhibition of secretion
Rabbit corpus luteum	Growth, steroidogenesis
Bovine corpus luteum	Luteolysis
Mammary cancer (estrogen-dependent)	Growth

The estrogen-dependent tumor that we examined is the adenocarcinoma of the mammary gland induced in Sprague-Dawley rats by administration of 7,12-dimethylbenz(*a*)-anthracene (DMBA) (9,10). It has been shown that many of these tumors will stop growing or will regress following estrogen deprivation, and that the tumor tissue contains significant concentrations of a cytoplasmic estrogen-receptor protein (11).

Question: Will $PGF_{2\alpha}$ administration to rats bearing such tumors effect a reduction in tumor content of estrogen receptor to the point where the tumor growth rate is slowed or reversed despite the presence of estrogen?

The first experiment employed six DMBA-treated rats bearing 14 palpable tumors. Tumor growth rates were monitored beginning shortly before initiation of prostaglandin treatment. Each rat received either five or eight sc injections of $PGF_{2\alpha}$ on an irregular schedule according to the protocol in table 3. Injections ended when the prostaglandin supply was exhausted.

At regular intervals, the palpable tumors were measured in their longest and shortest dimensions to the nearest millimeter. The product of these (tumor "area") is a fair measure of tumor size. At the end of the treatment and examination period the rats were

TABLE 3.—Schedule for injection of $PGF_{2\alpha}$ into rats bearing DMBA-induced mammary tumors*

Date of injection	Rat No.— 1	2	3	4	6	9
10/25	L	L	L	M	M	M
10/27	L	L	L	L	L	L
10/29	L	L	L	M	M	M
10/30	L	L	L	M	M	M
10/31	L	L	L	L	L	L
11/1	V	V	V	H	H	V
11/2	V	V	V	H	H	V
11/3	V	V	V	H	H	V
11/13	A	A	A	A	A	A

*$PGF_{2\alpha}$ in saline containing 15% gelatin administered sc. L = 400 μg/kg; M = 800 μg/kg; H = 1000 μg/kg; V = vehicle; A = autopsy.

autopsied and the tumors were rapidly dissected, weighed, and frozen in liquid nitrogen. These specimens were stored in a liquid nitrogen refrigerator until they could be analyzed for their content of cytoplasmic estrogen receptor.

The results are summarized graphically in figure 5. During the 12-day interval before drug injections began, the tumors were in a state of rapid growth. During this span of time, the median tumor area increased from 0.25 to 1.58 cm^2, and the sum of all tumor areas increased from 21.34 to 39.16 cm^2. The 10-day treatment regimen that followed depressed the median tumor area to 1.00 cm^2 and the total area to 28.33 cm^2, a decline of about 35% in each. Area measurements obtained during the subsequent 10-day posttreatment interval suggest that the tumors were able to resume growth after drug treatment was discontinued.

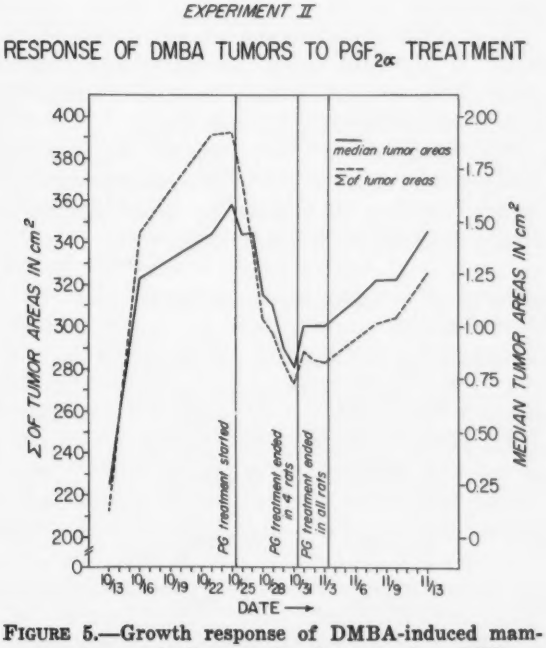
EXPERIMENT II
RESPONSE OF DMBA TUMORS TO PGF2α TREATMENT
median tumor areas
Σ of tumor areas
Σ OF TUMOR AREAS IN cm²
MEDIAN TUMOR AREAS IN cm²
PG treatment started
PG treatment ended in 4 rats
PG treatment ended in all rats
DATE

FIGURE 5.—Growth response of DMBA-induced mammary tumors in Sprague-Dawley rats given $PGF_{2\alpha}$ sc in 15% gelatin according to the dosage schedule in table 3. Six rats, 14 tumors.

A tumor that is ellipsoidal in shape has a volume proportional to its area raised to the 3/2 power. The reduction in cross section of 35% therefore represents a regression in tumor volume (and hence mass) of about 50%, achieved within a few days of treatment.

With the encouragement provided by the result described above, a second experiment was performed according to the following protocol, employing a generous supply of $PGF_{2\alpha}$ provided by Drs. James Weeks and John Pike of The Upjohn Company.

Fourteen rats bearing DMBA-induced mammary tumors received 1.5 mg/kg of $PGF_{2\alpha}$ sc daily in 15% gelatin for 10 days. The dose was divided (0.5 mg/kg in the morning, 1.0 mg/kg in late afternoon). Seven tumor-bearing rats received vehicle only on the same schedule. Autopsy was on the 11th day.

Before treatment rats were weighed and examined twice weekly. All tumors detectable by palpation were measured (long and short diam-

eter) to the nearest millimeter. During the 10-day treatment period, body weights and tumor areas were recorded daily. At autopsy, all tumors found (including several previously undetected by palpation) were weighed to the nearest milligram.

Over the time interval between DMBA treatment and autopsy, the body weight curves of the prostaglandin-treated and vehicle-treated rats were indistinguishable.

The graph in figure 6 shows the median tumor area for the treated group and for the control group, plotted versus the day the animals were examined. Area less than 0.25 cm^2 is too small to determine with acceptable accuracy and is recorded as less than 0.25 cm^2.

It is apparent from the graph that the tumor populations in the treated and control groups are similar until $PGF_{2\alpha}$ treatment begins, after which they diverge. Tumors in vehicle-treated rats continued to grow while those in prostaglandin-treated rats were markedly inhibited. The ratio of the median tumor area in treated rats and in control rats was close to unity when treatment began, then fell sharply to about 0.4. This effect seemed to be near its maximum after only 2 days of treatment and represents a decline in tumor volume of about 75% relative to controls.

There were about three tumors per rat when treatment began and at autopsy. Of the 21 tumors in vehicle-treated rats, ten (48%) increased in size by more than 10% during the treatment period. In the prostaglandin-treated rats, only four of 37 tumors (11%) increased in size, while about half of them substantially regressed.

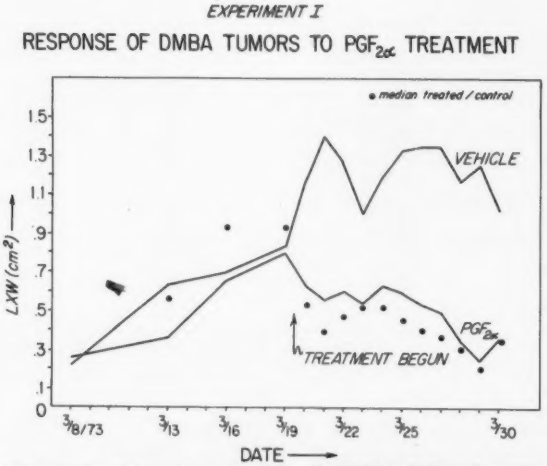

FIGURE 6.—Growth response of DMBA-induced mammary tumors in Sprague-Dawley rats given $PGF_{2\alpha}$ (1.5 mg/kg) sc in 15% gelatin each day for 10 days and in rats receiving vehicle only. The daily dosage was divided (0.5 mg/kg mornings and 1.0 mg/kg afternoons). Treated group: 14 rats, 37 tumors. Control group: 7 rats, 21 tumors.

The growth responses are summarized in table 4. By this crude categorization, about 37% of the tumors in these rats have exhibited significant prostaglandin sensitivity.

The weights of the tumors obtained at autopsy corroborate the prostaglandin-induced tumor atrophy indicated by area measurements. The tumor weights (mg) were:

TABLE 4.—Tumor population changes in response to daily administration of $PGF_{2\alpha}$ (1.5 mg/kg) for 10 days

	No. of tumors	No. of tumors— Regressing ($A_{n/1}$*<0.9)	Unchanged ($A_{n/1}$*>0.9,<1.1)	Growing ($A_{n/1}$*>1.1)
Treated animals	37	19 (51%)	14 (38%)	4 (11%)
Control animals	21	7 (33%)	4 (19%)	10 (48%)
Effect of treatment		+18%	+19%	−37%

*$A_{n/1}$ for each tumor is the ratio of final to initial tumor area over the 10-day treatment interval.

Animals	*No.*	*Median*	*Mean*
Control	21	615	1801
Treated	37	137	802

The median tumor mass at autopsy in the prostaglandin-treated rats is thus reduced 78% relative to that in control animals, in good agreement with the 75% reduction in median tumor volume calculated from the median areas in figure 6.

About two thirds of the tumors obtained in this experiment have been analyzed for their content of cytoplasmic estrogen receptor by published methods (12). The median estrophile concentration was 0.50 fM/mg in tumors from prostaglandin-treated rats and 0.48 fM/mg in tumors from vehicle-treated animals. These are indistinguishable. However, the concentration of estrophile relative to tumor size appears to have been altered by treatment, in a manner whose significance we have not determined. In both groups, there appear to be two populations of tumors distinguishable by their mass at autopsy. The small masses range from a few milligrams up to about 650 mg. The large tumors have a minimum weight of 1.05 g and range up to 11 g.

Of the 37 tumors obtained from prostaglandin-treated rats, 28 have to date been assayed for estrophile content. A scattergram plot of estrophile concentration versus tumor mass discloses no relationship between these variables. Similar scrutiny of the data after dividing the tumors into small (weight < 0.66 g) and large (weight > 1.3 g) groups revealed no relationships.

Of the 21 tumors obtained from vehicle-treated rats, 16 have been assayed for estrophile. Of these, eight are small (weight $<$ 0.62 g) and eight are large (weight > 1.05 g). Estrophile concentrations in the small tumors range from 0.23 to 0.80 fM/mg. In the eight large tumors seven have estrophile concentrations outside this range. In increasing order, they are 0.01, 0.01, 0.16, 0.17 . . . 0.50, 0.96, 1.01, and 1.45 fM/mg. Very high *and* very low estrophile concentrations thus appear only in the large tumors with one exception: mid-range estrophile concentrations appear only in the small tumors.

These experiments were undertaken to test the dual hypothesis that administering $PGF_{2\alpha}$ to rats bearing estrogen-dependent mammary cancer would attenuate the content of estrophile within the tumors and would thereby cause their regression. They have provided evidence that administering $PGF_{2\alpha}$ to rats bearing DMBA-induced tumors can cause a rapid and dramatic decline in tumor mass. However, they provide no evidence as to whether remission did or did not follow as a consequence of a reduction in the tumor content of estrophile.

In fact, the results contain no evidence that the estrophile content was reduced by the prostaglandin treatment, since the median receptor concentrations in tumors from control and $PGF_{2\alpha}$-treated rats are indistinguishable (0.48 vs 0.50 fM/mg).

During the design of the experiments, it was considered likely that large as well as small tumors obtained from control rats would be found to contain a spectrum of estrophile levels from very high to very low. We speculated that in treated rats the small tumor count would be increased at the expense of large tumor count, principally by depletion of the population of large tumors with high estrophile content (*see* fig 7). However, it appears that the number of tumors studied in such an experiment would have to be substantially larger in order to render that phenomenon demonstrable. Based on the variation we have observed in the estrophile content of control rats, our statistical consultants have estimated that approximately 100 animals per group would be required in this study in order to achieve a valid conclusion. We preferred not to undertake an experiment requiring 600 estrophile analyses and explored another approach.

In the diagram in figure 8, two possible pathways are proposed for the conversion of a population of prostaglandin-sensitive tumors into a new population with smaller median weight but with unchanged median receptor concentration. Path A describes the abrupt reduction of tumor content of estrophile by drug treatment, producing estrogen-dependent tumors markedly deficient in their ability to respond to estrogen. This leads promptly to

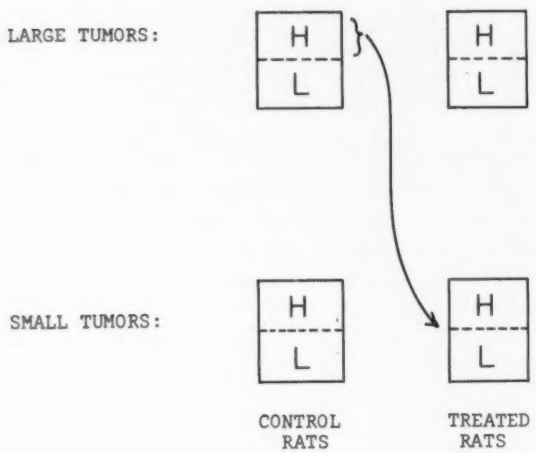

FIGURE 7.—Anticipated change in rat mammary tumor population following $PGF_{2\alpha}$ treatment. The arrow indicates a probable increase in the number of small tumors at the expense of the number of large tumors that have a high estrophile content.

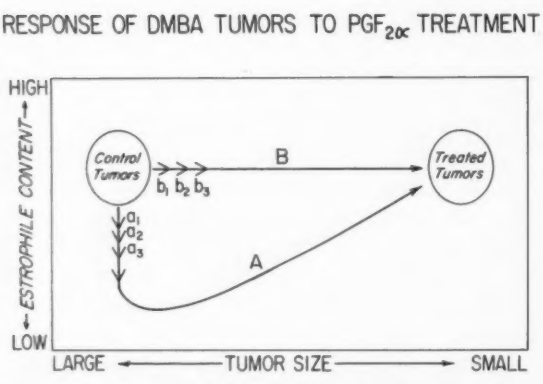

FIGURE 8.—Two possible pathways for the prostaglandin-induced change of state that a tumor population might traverse in being transformed into a group of smaller tumors that retain the original tissue concentration of cytoplasmic estrogen receptor.

atrophy and perhaps lysis of the affected cells, a reduction of tumor mass, and a return to the original estrophile concentration that exists in the surviving unaffected cells.

Path B presumes that an equal proportion of all cell types in the tumor are lysed by prostaglandin exposure, again producing a small tumor with the original estrophile concentration and without the intervening existence of a lower concentration. This would imply that all tumor cells have the same susceptibility to $PGF_{2\alpha}$ regardless of estrophile content.

The experiments that have been described provide information about the initial and final states but do not inform us regarding the path traversed. We have, however, recently performed an experiment we hope will distinguish between paths A and B. We have administered a single dose of $PGF_{2\alpha}$ to two groups of tumor-bearing rats. We killed one group after 12 hours and the second group after 24 hours. A vehicle-injected control group was killed to provide 0 time data. The tumors dissected at autopsy have been preserved for assay of their estrophile content. That ambitious task (120 analyses) is in progress, but adequate data are as yet not available. If the prostaglandin treatment initiates a change in tumor biology that traverses path A, we are likely to observe a progressive decline in the mean estrophile content of the tumors from 0 to 12 to 24 hours. This result would support the analogy I have drawn between the prostaglandin-induced regression of estrogen-dependent mammary tumors in rats and lysis of the estrogen-dependent corpora lutea in rabbits. If the prostaglandin effect on tumors traverses path B, we should find the estrogen receptor levels in the three groups to be indistinguishable.

We have thus begun a program of investigation in which several vital biochemical parameters are being studied in rabbit corpus luteum as a model tissue and in estrogen-dependent mammary cancer as a tissue of prime interest. The two tissue types are undergoing scrutiny in parallel, with the tumor studies of necessity remaining one or more "progress-points" behind the model tissue study.

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Prediction of Breast Cancer Response to Endocrine Therapy [1]

E. R. DeSombre,[2] S. Smith,[2] G. E. Block,[3] D. J. Ferguson,[3] and E. V. Jensen [2]

SUMMARY

Human breast cancers fall into two categories, those that contain significant amounts of estrogen receptor and those that do not. These can be distinguished by examination of a cancer specimen in vitro. Approximately one half of the primary tumors examined contain receptor, whereas with metastatic tumors receptor-lacking specimens predominate. In the case of metastatic disease, approximately three fourths of the patients with receptor-containing tumors may expect to benefit from endocrine therapy; the response rate is even higher with those patients in whom a receptor-containing primary cancer is still present. In contrast, patients whose cancers show no significant content of estrogen receptor have little chance of remission and probably can be spared the trauma of adrenalectomy or other endocrine ablation.

[Cancer Chemother Rep 58:513–519, 1974]

It is well established that hormone deprivation affords striking regression of advanced breast cancer in some but not in all individuals. Clinical experience has shown that somewhat less than one half of the younger patients can expect to benefit from ovariectomy, whereas a much smaller percentage (20%–25%) of postmenopausal patients with breast cancer respond to adrenalectomy or hypophysectomy. A method of predicting a priori which breast cancers are of a hormone-dependent type can enhance the usefulness of endocrine ablation by restricting its application to those individuals in whom it has a reasonable chance of success, thus sparing the majority of patients the trauma of major surgery that cannot help them and which renders them less able to tolerate alternative types of therapy. It appears that this objective may be attained by determining the estrogen-receptor content of an excised specimen of the tumor (1–5).

The two types of breast neoplasms, hormone-dependent and hormone-independent, have their counterparts in normal mammalian tissues, some of which require steroid hormones for their growth or function and some of which do not. An important characteristic of hormone-dependent or "target" tissues is their content of specific steroid-binding proteins, first recognized in the case of estrogens by the striking affinity of such tissues for tritiated estradiol or hexestrol, and characterized most informatively by sedimentation of the estrogen-receptor complexes in sucrose density gradients. On the basis of contributions from many laboratories (6,7) it appears that all classes of steroid hormones interact with their respective target tissues by a two-step

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[2] Ben May Laboratory for Cancer Research, University of Chicago, Ill.

[3] Department of Surgery, University of Chicago, Ill.

mechanism first elucidated for the estrogens, in which the hormone combines with an extranuclear receptor protein, inducing its translocation to the nucleus. Here the hormone-receptor complex accumulates in the chromatin and in some way initiates or accelerates biosynthetic processes, especially that of transcription. In the case of estrogens (8), migration to the nucleus is accompanied by conversion of the receptor protein to an active form, recognizable by a change in sedimentation properties as well as by the acquisition of an ability to bind to uterine nuclei and to stimulate their RNA polymerase activity.

Information about the interaction of estrogenic hormones with uterine tissue has provided a basis for comparing hormone-dependent cancers with autonomous cancers. Soon after the original observations of the characteristic affinity of target tissues for estrogenic hormones, Folca et al (9) injected tritiated hexestrol into patients with breast cancer just prior to adrenalectomy and observed that the uptake of radioactive hormone by the tumor, as compared to that of the skeletal muscle, was greater in four patients who experienced remissions than in six patients who did not respond. Later studies in the same institution (10) found less precise correlation of estradiol uptake with response to endocrine therapy, although patients with low uptake tended not to respond favorably. Numerous investigators have confirmed the ability of some human breast tumors to concentrate estrogenic hormones in vivo (11–14) and in vitro (1, 15–18), as well as the presence of receptor protein in the cytosol fraction of a tumor homogenate (1–5, 19–24).

In contrast to their nondependent counterparts, certain hormone-dependent mammary tumors in the rat (25–33) and in the mouse (34,35) were found to resemble uterine tissue in their specific binding of estradiol, both in vivo and in vitro, and in their content of extranuclear receptor protein that serves as the precursor of the estradiol-receptor complex found in the nucleus (32,33).

The foregoing indications of similarity in estrogen binding by hormone-dependent mammary tumors with that shown by estrogen target tissues suggested that the receptor content of human breast cancers, determined in vitro, might provide an index for predicting which patients might benefit from ablative endocrine therapy (29). In 1966, we began a study of tumor specimens from individuals with metastatic cancer who were about to undergo endocrine therapy, as well as tumor specimens from mastectomy patients in order to characterize the primary tumors for future reference in case metastases might later appear.

ESTROGEN BINDING BY HUMAN CANCERS

In the earlier experiments, the estrogen affinity of human breast cancer specimens was determined by incubating 0.5-mm slices of the tumor tissue at 37 C in dilute (0.1 nM) solutions of tritiated estradiol in Krebs-Ringer-Henseleit buffer in the presence and absence of an inhibitor of specific binding, either nafoxidine (36) or Parke-Davis CI-628 (37). This uptake inhibition procedure provides a convenient means for characterizing breast cancers, but it is subject to two limitations. First, the tumor specimen must be sufficiently large (> 0.5 g) to provide five tissue slices for each time point, so that variation due to the heterogeneity of the cancer tissue can be minimized. Second, only fresh tumor specimens can be used; freezing renders the cells permeable to the extranuclear receptor protein so that it is extracted into the incubation medium and is not available for translocation of the hormone to the nucleus.

It was later found that direct estimation of the extranuclear receptor by sucrose gradient ultracentrifugation of the high-speed supernatant or cytosol fraction of a tumor homogenate provides a method of tumor characterization that is applicable to small (200-mg) samples of either fresh or frozen tissue. As illustrated in figure 1, the weighed tumor specimen was immersed in liquid nitrogen and shattered in a Thermovac Autopulverizer (Thermovac Industries Corp, Copiague, NY).

The residual tissue powder was homogenized with efficient cooling in four volumes of 10 mM tris buffer, pH 7.4, containing 0.5 mM dithiothreitol (38, 39), using a Polytron PT-10 tissue disintegrator (Brinkmann Instruments, Inc) with two or three 10-second homogenization periods, each followed by a 50-second cooling

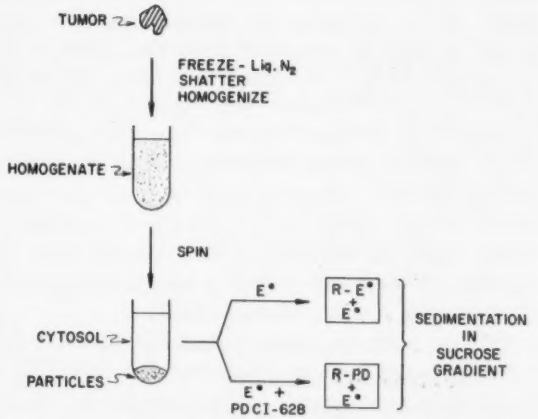

FIGURE 1.—Preparation of the specimen for determination of receptor content.

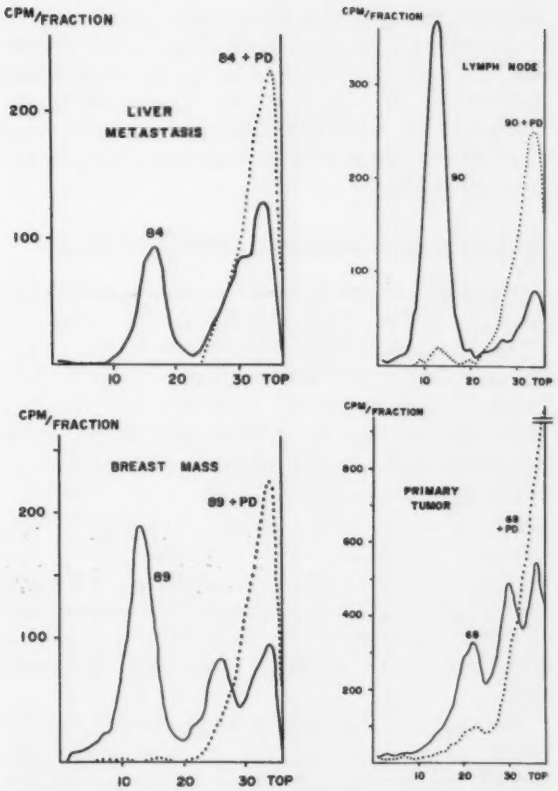

FIGURE 2.—Typical sedimentation patterns in sucrose gradients of positive human breast cancer cytosols containing 0.5 nM tritiated estradiol (2 nM for No. 68) in the absence (solid line) or presence (dotted line) of 0.2 μM Parke-Davis CI-628. Some tumor cytosols show only 8S complex in low-salt gradients; others show varying amounts of subunit as well. Negative tumor cytosols show patterns similar to those illustrated for positive tumors plus Parke-Davis CI-628, with no significant effect of the inhibitor. [Reproduced from Jensen et al (1).]

period. The homogenate was centrifuged at 2 C for 30 minutes at 210,000 × *g* to precipitate the particulate matter. Two 150-μl portions of the cytosol fraction were removed and treated with 50 μl of either buffer alone or of buffer containing 1 μM Parke-Davis CI-628. After 10 minutes, 50 μl of buffer containing 2.5 nM tritiated estradiol was added to each mixture. In earlier experiments, illustrated in figure 2, estradiol-6, 7-^{3}H of specific activity 57 curies/mmole was used; recent experiments employed estradiol-2,4,6,7-^{3}H of specific activity 109 curies/mmole (New England Nuclear Corp). After mixing and standing for 30 minutes in the cold, a 200-μl portion of each mixture was layered onto 3.4 ml of a 10%–30%-sucrose gradient containing 10 mM KCl and 1 mM EDTA in 10 mM tris buffer, pH 7.4, and centrifuged at 2 C for 16 hours at 250,000 × *g*. Successive 100-μl fractions were collected from the bottom via an 18-gauge needle by displacement with paraffin oil, and were counted for radioactivity in a toluene-Triton X-100 scintillation medium.

As illustrated in figure 2, the estradiol-receptor complex of some human breast cancer cytosols sediments in a low-salt sucrose gradient entirely in an 8S form (Nos. 84, 90) while with other cancers the complex exists partly in a 3S–4S form (Nos. 68, 89). This may represent the steroid binding unit of the 8S receptor which in the case of the human uterus (40) is converted enzymatically during tissue processing to a form that remains as a smaller entity in low-salt gradients. Both of these specific sedimentation peaks can be distinguished by their sensitivity to Parke Davis CI-628 from weak, nonspecific (4.6S) binding to serum proteins that is seen with some cancer specimens. With negative tumor specimens, identical sedimentation patterns are observed in the presence or absence of the inhibitor, with the ra-

dioactive estradiol appearing either at the top of the tube or, if sufficient serum proteins are present, partly bound in the 4.6S region.

Cancer specimens, beginning with patient No. 47, were all evaluated by the sedimentation procedure. In those cases where sufficient sample was available, slice uptake also was carried out, with excellent agreement between the two methods.

RESULTS AND CLINICAL CORRELATIONS

As summarized in table 1, the 251 primary tumors studied were equally divided between those that contained receptor and those that did not, with a small number of borderline cases. The presence of metastases in the patient did not appear to alter this ratio. With 142 metastatic tumors examined, a higher proportion of negative patterns was observed. In most cases where more than one tumor specimen (primary and metastasis, or two or more metastases) was obtained from the same patient, all samples showed similar patterns; but in three instances, both positive and negative metastases were obtained from the same patient.

Because of the higher incidence of remissions after ovariectomy in premenopausal breast cancer patients than after adrenalectomy in older patients, one might expect that receptor-containing tumors would be more prevalent in the younger-age group. Based on the cases studied so far, this does not seem to be so; if anything, the opposite is true. Although both types of tumors were found in all age groups, the median age for the 116 patients with positive primary tumors was 61 years (range 30–85), as compared to a median age of 54 years (range 28–86) for the 114 patients with negative tumors. These results are in agreement with our previous conclusions based on a smaller number of cases (1,3).

Of the patients with metastatic disease, 86 received some type of endocrine therapy under conditions that permitted evaluation of clinical responses. In most instances, treatment commenced at the time the tumor was characterized, but in a few cases it was carried out either before or after the tumor sample was taken for study. Of 43 patients whose cancers showed negative patterns, only one showed remission after endocrine therapy, as did one of six patients with borderline patterns (table 2). In contrast, 27 of 37 patients whose cancers contained receptor experienced objective remissions. The foregoing results are in general agreement with our earlier observations (1,3), as well as with the findings of others

TABLE 1.—Occurrence of estrogen receptors in human breast cancers*

	Tumor specimens			
	Positive	Borderline	Negative	Mixed†
Primary tumors	116	21	114	
Without metastases	94	16	96	
With metastases‡	22	5	18	
Metastatic tumors§	53	9	77	3

* Presence of receptor determined by estradiol uptake in tumor slices and/or sedimentation peak on sucrose gradient centrifugation of cytosol treated with tritiated estradiol in the presence and absence of a specific binding inhibitor.

† Patients from whom both positive and negative specimens were obtained.

‡ Of the patients whose primary tumors were accompanied by metastases, 28 received endocrine therapy and are included in table 2.

§ Both positive and negative specimens have been observed with metastases in the lymph nodes, skin, liver, adrenal, chest wall, bone, omentum, and lung. So far only positive metastases have been found in the ovary and aortic nodules and negative metastases in the brain and skull, although the number of specimens examined is as yet too small to be representative.

TABLE 2.—Summary of objective remissions* †

Endocrine therapy	Tumor specimens		
	Positive	Borderline	Negative
Ablation (73 patients)			
Adrenalectomy	18/26	0/3	1/18
Hypophysectomy	3/4		0/7
Oophorectomy	2/2	1/2	0/11
Hormone (13 patients)			
Androgen			0/1
Estrogen	2/2	0/1	0/4
Antiestrogen	1/1		0/1
Estrogen plus progestin	1/2		0/1
Total (86 patients)	27/37	1/6	1/43

* In 28 patients the primary tumor was examined, whereas in 58 patients the primary tumor was absent and one or more metastases were taken for study.

† Numbers represent No. of responses/total No. of patients treated.

(2,4,5). Most, but not all, patients with receptor-containing breast cancers will respond to endocrine therapy, but there is little chance of benefit if the cancer lacks receptor.

The failure to obtain a response in some patients with positive patterns is understandable if one considers that it is possible to have a mixed population of positive and negative metastases in the same individual. In such cases, the pattern observed may be either positive or negative, depending on which metastasis is obtained for study, but the endocrine treatment will fail because the negative metastases will be unresponsive. Regression of those lesions that contain receptor could explain the fact that three of the ten patients with positive tests, who did not show objective remission after endocrine ablation, did experience subjective remission with relief of pain.

It is noteworthy that, of the 28 patients with metastatic disease whose primary tumors were used for study, 12 of 13 with positive tests showed response to endocrine therapy, whereas only one of five with borderline tests and none of ten with negative tests showed response (table 3). Although more cases must be studied before definitive conclusions can be drawn, these results suggest that a receptor-containing primary tumor, when present, provides a better prognosis for favorable response to endocrine therapy than does a receptor-containing metastasis. Although the significance of this phenomenon is not clear, it is conceivable that in these patients where metastatic disease accompanies the primary tumor, the spread of the cancer from its initial site has been especially rapid, so that there has been less time for metastases from a positive primary tumor to lose their hormone dependency.

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examined*

TABLE 3.—Summary of objective remissions when primary tumor was

Endocrine therapy	Tumor specimens		
	Positive	Borderline	Negative
Ablation (22 patients)			
Adrenalectomy	8/8	0/2	0/4
Hypophysectomy	1/1		0/2
Oophorectomy	2/2	1/2	0/1
Hormone (6 patients)			
Androgen			0/1
Estrogen		0/1	0/1
Estrogen plus progestin	1/2		0/1
Total (28 patients)	12/13	1/5	0/10

* Numbers represent No. of responses/total No. of patients treated.

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Adrenal Steroids in Leukemia and Lymphoma [1]

Martin J. Cline [2]

The adrenal glucocorticoids have two major roles in therapy for leukemias and lymphomas: (*a*) direct destruction of neoplastic cells, thereby reducing the tumor mass; (*b*) treatment of complications of malignant disease, including hemolytic anemia, thrombocytopenia, and hypercalcemia. Therefore, two different indications for the use of corticosteroids in clinical cancer therapy must be considered—primary treatment of neoplastic disease and supportive or ancillary therapy. In primary therapy steroids are usually used in combination with other drugs; in supportive treatment they may be used alone.

In malignant hematologic disease steroids are largely restricted to the treatment of "lymphoid" tumors (acute and chronic lymphatic leukemias, lymphomas, and multiple myeloma). Their use in acute myelocytic leukemia is still equivocal and they have no place in the management of chronic myelocytic leukemia.

CELLULAR EFFECTS

Although steroids have been known since 1944 to exert an influence on the structure and function of lymphoid cells of several species (1,2), the mechanism of their lympholytic effect is still uncertain. This cytolytic effect is probably exerted on distinct subpopulations of lymphoid cells. Wira and Munck observed binding of labeled cortisol to thymocyte nuclei (3). From the observed effect of corticosteroids on many populations of chronic lymphocytic leukemic cells and myeloma cells one would predict that these agents also affect B-cell populations.

In vitro, steroids can inhibit RNA and protein synthesis in lymphoid cells (4, 5), and it has been suggested that this inhibition ultimately results in the destruction of these cells in vivo (5). However, this mechanism has certainly not been proved. Interference with RNA synthesis may occur via inhibition of RNA polymerase (6).

Another possible mechanism by which steroids may produce lympholysis is by means of blocking glucose transport and/or phosphorylation (7) and perhaps the transport of other compounds. This interference may conceivably be related to the interaction of steroids with the plasma membrane of lymphoid cells. A number of studies suggest that the toxic effect of glucocorticoids may be mediated by such an interaction (7–12).

Glucocorticoids are known to exert their effect on lymphoid target cells whether or not these cells are in a proliferative state as, for example, after phytohemagglutinin stimulation. Steroids are said to be "cell-cycle nonspecific" in their effect on lymphoid target cells. Steroids are distinguished in their mode of action from "cell-cycle specific agents" such as methotrexate, whose major cytotoxic effects are exerted during the period when the neoplastic cell is synthesizing DNA.

The metabolic effects of steroids on lymphoid cells may result from either glucocorticoid actions which can be demonstrated at physiologic concentrations and are triggered by the interaction of steroid with glucocorticoid-specific receptors in the cell cytoplasm or nonspecific effects of steroids which are unrelated to glucocorticoid potency, develop only at high concentrations, and may not be physiologically or pharmacologically significant.

Many hormonally active tissues contain receptors for steroids. For example, estrogen receptors have been identified in uterine and breast tissues, and androgen receptors exist in

[1] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[2] Division of Hematology-Oncology, University of California Center for the Health Sciences, Los Angeles.

the prostate. Similar cortisol receptors have been demonstrated in lymphoid tissues (13). An important recent observation is that the responsiveness of lymphoid tumors to glucocorticoids may be determined by the presence or absence of these specific steroid receptors in the cell cytoplasm (14,15). This observation obviously has important implications for the treatment of lymphoid neoplasms, since glucocorticoids are used extensively in the treatment of this class of malignancy. Baxter et al and Claman have recently reviewed the tissue effects of glucocorticoids (16,17).

USE IN CHEMOTHERAPY

All glucocorticoids appear to be similar in their antineoplastic action in hematologic malignancies when used in comparable dosages. The general use of prednisone and prednisolone in these conditions has been dictated by their relative freedom from undesirable side effects rather than by any unique antitumor property of these compounds. Furthermore, when neoplastic disease becomes resistant to one glucocorticoid, it is resistant to all glucocorticoids. The schedule of administration of steroids in lymphomas and leukemias does not appear to be critical provided administration is frequent enough to maintain effective plasma levels, ie, prednisone in divided oral doses totaling 40–100 mg/m²/day or an equivalent intravenous dose.

ACUTE LEUKEMIA

Shortly after their introduction into clinical medicine, corticosteroids were shown to be effective in acute lymphocytic leukemia (synonym, acute lymphoblastic leukemia [ALL]). For this discussion, this disease entity may be considered as comprising ALL occurring at any age and acute undifferentiated leukemia occurring in children and young adults.

Although steroids are now used only in combination with other drugs in the treatment of ALL, several important observations were made during the "single-drug era" of leukemia therapy: (*a*) steroids alone used daily in the first course of treatment produce a complete remission rate of between 45% and 65% (18); (*b*) when steroids are used a second time after the disease has regressed, the rate of remission induction falls to about 25% (19); (*c*) a daily dose of steroids is superior to an intermittent schedule in remission induction (20) (this phenomenon may be related to the necessity of maintaining a tumoricidal blood level); (*d*) steroids are better when used in remission induction than when used as single agents in remission maintenance (21).

In the early 1960s it was shown that steroids used in combination with other agents were superior to drugs used singly in the treatment of ALL. The most successful early combination proved to be prednisone, 2–4 mg/kg/day (or 40–100 mg/m²/day) with vincristine. This combination produced remission in greater than 80% of children with ALL (22). This combination is still the best "standard" for remission induction (23). In some protocols other drugs such as daunorubicin, 6-mercaptopurine, or methotrexate are added. With such multiple-agent combinations, the rate of remission induction may rise to 88%–100% (22,24,25). Such drugs may be considered when the simple combination of vincristine and prednisone fails.

After remission induction, vincristine and prednisone are used intermittently in some studies for remission maintenance. The value of such "reinduction pulses" is under investigation and is still uncertain (23,26).

From the foregoing one can conclude that steroids used in conjunction with drugs having other modes of action are extremely useful in remission induction of ALL and perhaps in remission maintenance. Corticosteroids are also among the few agents used in the treatment of ALL that have little or no myelosuppressive effect.

The use of corticosteroids as effective agents in acute myelocytic leukemia (AML) is less certain. Used alone they induce remissions (complete and partial) in 20% or less of cases in children (20,27) and in 15% or less of cases in adults (22,28). There is some question whether steroids alone ever benefit adult patients with AML (29,30).

CHRONIC LEUKEMIA

Adrenal corticosteroid use in leukemias is restricted to chronic lymphocytic leukemia

(CLL). Presently, the usual objective of therapy in this disorder is to produce a complete remission, if possible. If complete remission is not achievable, one attempts to improve the quality of life by treating disfiguring or obstructing collections of lymph nodes, or by alleviating complications such as thrombocytopenia or hemolytic anemia. Steroids are of greatest use in the treatment of such complicating cytopenias (31). These agents are generally used briefly in large doses until the complications are controlled. The steroids are then quickly reduced to the minimal dose levels necessary to control symptomatology.

Steroids are also used in the primary treatment of CLL with the intent of reducing lymphoid mass. Often steroids are used in combination with alkylating agents in intermittent "pulses." Under two circumstances steroids may be preferable to alkylators alone as primary therapy: when disease is resistant to alkylating agents, and when the marrow is densely packed with lymphocytes and a complicating granulocytopenia or thrombocytopenia exists. In such circumstances corticosteroids have a lympholytic effect with little myelosuppression (31,32).

Although they are lympholytic, the initiation of steroid therapy may be associated with a transient increase in the peripheral lymphocyte count, sometimes to very high levels. This leukocytosis occurs at a time when the central lymphoid tissues are shrinking (31).

Occasional reports suggesting that long-term high-dose steroid treatment of CLL is useful have not been confirmed. Obviously such long-term therapy is undesirable for a patient who already has an increased susceptibility to infection. Consequently, in CLL steroids are reserved for brief management of primary disease or of secondary complications.

LYMPHOMAS

The activity of steroids against lymphomas (including Hodgkin's disease) appears to be comparable to their activity against CLL (33, 34). Used alone, steroids produce worthwhile objective responses in about two thirds of patients (35); however, remissions achieved with steroids used alone are rarely complete and are often of short duration (36). Treatment is often attended by severe complications in an impaired host.

When lymphoma is widely disseminated, treatment with multiple chemotherapeutic agents has the best chance of producing long remissions (37–40). Such drug combinations invariably include steroids along with drugs with other modes of antitumor action: cyclophosphamide or mechlorethamine, vincristine, and procarbazine (table 1). In such combinations, prednisone is generally given intermittently in daily doses of 40–100 mg/m^2/day in 5- or 7-day "pulses."

Steroids used as single agents may be useful in the treatment of lymphomas when hemolytic anemia or thrombocytopenia develops and myelosuppression precludes other therapy. Steroids are also useful in combination with irradiation, cytotoxic drugs, and sometimes surgical laminectomy in the treatment of lymphomatous spinal cord compression.

TABLE 1.—Combinations of steroids with other drugs in the treatment of lymphoma

Drug combination	Disease	Reference No.
Cyclophosphamide, vincristine, prednisone	Advanced lymphosarcoma	37
Mustard, vincristine, prednisone, procarbazine	Advanced Hodgkin's disease	38
Cyclophosphamide, vincristine, prednisone	Lymphosarcoma, histologic lymphoma	39
Cyclophosphamide, vincristine, prednisone	Hodgkin's disease, lymphosarcoma, histologic lymphoma	40

CONCLUSIONS

Steroids used alone or in combination with other types of drugs have an important place in the treatment of ALL and CLL, disseminated Hodgkin's disease, and other lymphomas. In such diseases, steroids are used for their lympholytic effects that are achieved without myelosuppression. As single agents, steroids may be useful in treating complications of lymphoma and lymphocytic leukemia, including hemolytic anemia, thrombocytopenia, and hypercalcemia.

LIST OF COMPOUNDS

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Prednisolone: NSC-9900; pregna-1,4-diene-3,20-dione-, 11β,17,21-trihydroxy-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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The Role of Radiation Repair Mechanisms in Radiation Treatment Failures [1]

J. E. Byfield [2]

SUMMARY

The repair of radiation-induced DNA damage contributes significantly to the treatment-failure rate after X-ray therapy for human cancers. Since tumor-cell number is a variable independent of the cell numbers which constitute the normal tissues in any given treatment volume, inhibition of the repair of X-ray-induced DNA damage in tumor cells is of increasing theoretic advantage during X-ray therapy for advanced cancers, particularly for those of the epidermoid-transitional cell group in which the cure rate and the local control rate are closely related. Progress in the development of rational combinations of drugs and radiation has been hampered by a lack of precise knowledge as to the molecular basis for radiation-induced cell lethality. X-ray-induced lesions in DNA include base damage, single- and double-strand DNA strand breaks, and the formation of a variety of heteroadducts. Each of these lesions can be repaired but there is no general agreement as to the relevance of the various repair steps in the recovery of cellular clonogenic potential during split-dose experiments. Only the latter (Elkind-Sutton repair) can be related directly to clinical failures. During a series of recent experiments, we have studied in detail several of these molecular repair modalities in the context of the eliciting X-ray dose and have examined the effects of a variety of useful anticancer drugs on each repair modality. The effects of agents capable of repair inhibition were also studied for their effect on radiation survival curves and for recovery during split-dose experiments. Target cell lines from human cervical, bladder, and prostatic cancers have been studied. It can be shown that X-ray radiosensitization is possible with most agents which bind to cellular DNA. Since such agents (eg, actinomycin D, adriamycin, etc) inhibit both repair replication and DNA strand-break rejoining, it is not certain which molecular mechanism is responsible for the radiosensitization effects. However, it is clear that both cellular radiosensitization and inhibition of molecular repair occur at drug concentrations attained in vivo, and that this approach may prove useful in clinical cancer therapy and in screening for new agents.

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[2] Radiology Department, Harbor General Hospital, Torrance, Calif.

Radiation therapy is the oldest of the "new" methods of cancer therapy, "new" in that it originated shortly after the discovery of ionizing radiation early in this century and "old" in that it has been long accepted as the optimal means for curing certain forms of cancer. Treatment methods now in use have evolved empirically and only recently have radiologists acquired some insight into the mechanisms by which a population of malignant cells can be eliminated without coincident and unacceptable damage to the surrounding normal tissues. It seems worthwhile at this time to emphasize certain aspects of the radiobiologic properties of benign and malignant cells since there appear to be theoretic reasons for predicting that current X-ray therapy results can be improved. In particular, the converging boundaries of chemotherapy and X-ray therapy suggest that interactions based on a variety of radiobiologic phenomena may be fruitfully exploited in the near future. The topics considered in this paper deal exclusively with the radiobiologic response of mammalian cells existing without hypoxia and with genomes containing the nucleotides that normally exist in nature. The problem of eliminating in vivo hypoxia and the potentiality for increasing cellular X-ray sensitivity by analog base substitution are omitted *only* because they are dealt with in detail elsewhere in these proceedings(1,2).

Before discussing the potential for beneficial drug/X-ray interactions, it is important to remind ourselves of the uniqueness of the two fundamental requirements for the radiocurability of a tumor. First, for a tumor to be curable by X-ray therapy alone it must be localized at the time of initial treatment. This requirement, by and large, omits by definition several of the common human cancers such as those of the lung and breast since dissemination in the early stages characterizes most of these cancers. The second fundamental requirement for radiocurability stems directly from the first. For a patient to be cured by X-ray therapy, a tumor obviously must receive a radiocurative dose: however, the dose that can be delivered is usually determined not by tumor cell radiosensitivity but by the tolerance of the normal tissue volume containing the cancer. Normal tissue tolerance is a complicated function of treatment volume and fractionation (3,4). A precise understanding of each of these factors for any given cancer is necessary before any other parameter can be manipulated. The recent impressive results with Hodgkin's disease (5) illustrate the constant need to refine our techniques in the light of these two critical criteria; the improvements in treating Hodgkin's disease have resulted from an increased awareness of the biology of the disease and of the interaction between normal tissue repair mechanisms rather than any radical new departure in therapeutics.

If one considers the list of common human cancers eligible for radiocurability within these two heuristic restraints it is apparent that the list is comprised mainly of the *epithelial* tumors of the upper respiratory tract and the lower urogenital tract (table 1). To this list may be added the central nervous system (CNS) glioma group which also usually kills by local-treatment failure, and a few other cancers such as ovarian cancer in which systemic dissemination occurs relatively late. In aggregate, this group includes at least 34% of the cancers seen in the United States, a fact not always appreciated because of their disparate anatomic origins.

If we limit our attention to this group of tumors, it is then appropriate to ask ourselves why X-ray therapy fails to cure all of the patients suffering from these cancers? Certainly it is well established that 5-year X-ray therapy cures are commonly reported (6) for some of them (eg, uterine cervix and early larynx cancer). The most common explanation for X-ray therapy failure is that the cancer is "too far advanced" locally when first seen. It is to this statement that the remainder of this communication is directed since I believe that the response that the tumors are "too far advanced" obscures within itself the essential nature of current radiation-treatment failures as well as the potential for future radiation therapeutic successes. In fact, radiation-treatment failures derive from complex sources including tumor volume, stem cell number, degree of differentiation, hypoxia,

TABLE 1.—Cancers typically lethal in a majority of cases because of local-treatment failure

Site	Incidence among all cancers (%)	5-Year survival rate (%)
Lip	1.2	87
Tongue	1.2	32
Salivary glands	1.2	88
Floor of mouth	0.7	43
Other oral sites	0.8	45
Mesopharynx	0.7	29
Nasopharynx	0.3	28
Hypopharynx	0.4	17
Esophagus	1.5	3
Stomach	4.5	12
Liver	0.4	3
Gallbladder	1.1	7
Pancreas	2.4	1
Nasal cavities	0.4	40
Larynx	2.0	55
Cervix uteri	4.8	60
Ovary	2.4	32
Vulva	0.4	62
Vagina	0.2	36
Penis	0.2	69
Bladder	4.6	56
CNS	2.6	28
Total	34	

doubling time, and cellular repair capacities, each of which contributes to individual failures through interaction with the other variables. Since the means by which each of these variables can be beneficially controlled by manipulations external to the X-ray therapy series per se differ considerably, an understanding of their origins and mechanisms is necessary before improvements can be expected. It is appropriate, therefore, to review briefly the contributions that radiobiologists have made in these areas over the past 2 decades and to consider which variables can be subjected to study in the current clinical setting.

Radiobiologic Parameters of Mammalian Cells and Tissues and Their Implications

The development of the quantitative in vitro clonal assay by Puck et al (7) led almost immediately to a wealth of subsequent studies on mammalian cell survival and clonogenic potential after graded X-ray doses (8). The result of these studies is the well-known X-ray survival curve illustrated in figure 1. The major parameters of interest on such a curve are the slope (1/e, termed the D_o, which measures approximately 37% survival on the exponential portion of the curve), the extrapolation number (Y intercept for zero dose), and the D_q or quasi-threshold dose, the dose at which the back extrapolate intersects the 100% survival line. The D_q is the best current measure of the width of the shoulder region (9). The D_q and the shoulder region are thought to be of great importance, since they reflect the "inefficiency" of X-rays in the clinical dose range for cell killing. In the argot of radiobiology, the extent of the shoulder region of the survival curve is equated with the capacity of each cell type to accumulate and repair sublethal damage (8). The implications of this phenomenon were first clearly demonstrated by the experiments of Elkind and Sutton (10), who examined the effect of splitting a single dose (sufficient to reduce survival to a level on the exponential portion of the curve) into two portions with a progressively greater time

period between the first and second fractional dose (*see* insert to fig 1). When such a study is carried out it has, thus far, invariably been found that survival rapidly increases to a maximum substantially greater than that resulting from the single equivalent dose. It is now generally believed that Elkind-Sutton or split-dose repair and the shoulder on the survival curve originate from similar cellular repair mechanisms. Whether or not they are molecularly identical is not known. The magnitude of these repair mechanisms is not always appreciated, particularly because cell repopulation occurs coincidentally in both the normal and the malignant components of the treatment volume. Its importance can be illustrated by comparing the potential of a standard X-ray treatment series to kill a theoretic cell population that is either "resistant" to X-rays (eg, having a D_o of 200 rads) or "sensitive" (having a D_o of 100 rads). In the former case, a treatment regimen of 200 rads per day 5 days per week for 7 weeks could kill a cell population initially containing 4.5×10^{14} cells, providing no repair and no repopulation takes place. In the case of the sensitive cell population lacking both repair and repopulation, the same treatment series could eliminate an initial population of 2×10^{36} cells. Since this latter cell mass is equivalent to about 3×10^{22} human body masses (each containing 7×10^{13} cells), it is obvious that repair and repopulation vastly overshadow the initial tumor volume if total cell number were the only variable in cancer-treatment failures. This type of calculation illustrates the phenomenal capacity to repair X-ray damage present in both radiosensitive and radioresistant tissues. It also illustrates the effect that a difference in D_o or survival curve slope can have on cell killing. In the absence of other factors a difference of 100 rads in D_o yielded a difference of over 10^{22} cells killed. This cell number is equivalent to about 10^{10} human epidermoid cancers at the time of average initial clinical presentation. Since the difference in volume of an early versus an advanced epithelial cancer is seldom more than one order of magnitude, *it is clear that repair mechanisms vastly outweigh primary tumor volume in determining radiocurability.*

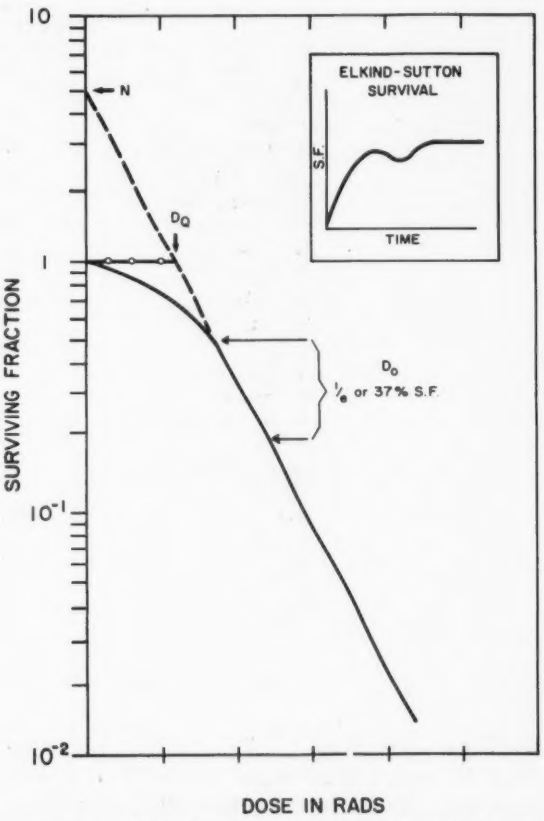

FIGURE 1.—Idealized radiation survival curve for mammalian cells in which cell survival (colony formation) is plotted against X-ray dose in rads on a semi-log plot. Pertinent parameters (*see* text under Radiobiologic Parameters of Mammalian Cells and Tissues and Their Implications) are the D_o or 1/e on the exponential portion of the curve, the extrapolation number (N), and the quasi-threshold dose or D_q which is an estimate of the repair capacity of the cell line. The insert shows the rising curve for cell survival found when a dose on the exponential portion of the curve is divided into two approximately equal doses with a variable time interval between the two doses; survival increases indicating the repair of sublethal damage. *See* ref 8 for a complete discussion of these topics.

Calculations such as the above are artificial because they ignore the processes operative in vivo which lead both to treatment failures and to the restoration of normal tissue integrity. However, it is perhaps worthwhile to follow the clues offered by this preliminary analysis

and to examine the effects of a typical X-ray therapy sequence when both the repair of sublethal damage and cellular repopulation are present. Figure 2 illustrates the measured survival curves for normal capillary endothelium (redrawn from data of Reinhold and Buisman, ref 11) and for a representative epithelial tumor line growing in tissue culture (averaged from the survival curves of three cervical epidermoid lines under study in our laboratory, ref 12); these are shown respectively as solid lines A and B in figure 2. The dotted lines are theoretic survival curves for cells with equivalent radiosensitivities (a D_0 of 170 rads for the capillary endothelial cells in vivo and 155 rads for the epidermoid cancer cells in vitro), each shifted towards the Y axis sufficient to eliminate the shoulder on curve B which is equivalent to eliminating repair to the same arithmetic degree in each line. From these four curves the surviving fraction for a typical clinical dose of 200 rads can be estimated. The minimum generation time for our cervix lines in culture is about 24 hours, and it seems reasonable to assume that normal tissues can attain at least similar reproductive rates when stimulated in vivo. Using these factors one can then calculate the effect of 1 week's treatment consisting of 200 rads daily for 5 days followed by a 2-day (weekend) rest, either with or without repair of sublethal injury, with or without cellular reproliferation, or lacking both. Such computations are given in tables 2 and 3. Several interesting observations appear (table 2): (*a*) if both repair and repopulation occur there is no chance of controlling the cancer with this regimen since the tumor mass on Day 8 is greater than on Day 1; (*b*) if repair of sublethal damage does not take place, the malignant cell population can be reduced by almost two logs with no effect on normal cell number. In a rapidly growing tumor, repopulation dominates repair, but repair must also be controlled to obtain a differential effect; (*c*) on the other hand (table 3), if proliferation is blocked without blocking repair, normal tissue cell killing by about 67% occurs but the tumor cells are killed to a much greater extent (about two orders of magnitude); and (*d*) if both repopulation and repair are blocked normal cell survival is reduced to 22%, while tumor cell killing now exceeds three logs.

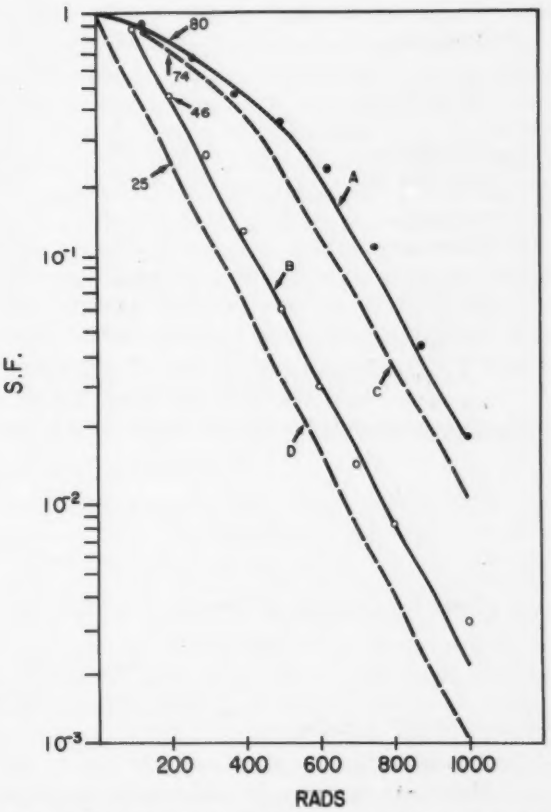

FIGURE 2.—Measured radiation survival curves for capillary endothelium (normal tissue equivalent) and cervix cancer cells. Solid line A shows survival for capillary endothelium in vivo (redrawn from ref 11) while line B shows survival for cervix epidermoid cancer cells in vitro (*see* text). Line C (dotted) shows the calculated survival curve for capillary endothelium when shifted to the left the same degree as for the cervix cells, sufficient to eliminate the shoulder on curve B. The shifted curve for the cervix cancer cells in line D, the shift to the left indicating no repair capacity ($D_q = 0$, the curve passing through the 100% survival point only at the zero-dose level). Numerals indicate the measured or estimated surviving fraction after a 200-rad dose for each curve; these values are the same as those used in tables 1 and 2 for calculation of single-dose killing capacity.

TABLE 2.—One week of X-ray therapy: 200 rads for 5 days, proliferation with and without repair*

		Repair active		No repair	
		Cancer cells	Normal cells	Cancer cells	Normal cells
Day	*Factor:*	*0.46*	*0.80*	*0.25*	*0.74*
Monday-b		1.0×10^9	1.0×10^9	1.0×10^9	1.0×10^9
Monday-a		4.6×10^8	8.0×10^8	2.5×10^8	7.4×10^8
Tuesday-b		9.2×10^8	1.0×10^9	5.0×10^8	1.0×10^9
Tuesday-a		4.2×10^8	8.0×10^8	1.25×10^8	7.4×10^8
Wednesday-b		8.4×10^8	1.0×10^9	2.50×10^8	1.0×10^9
Wednesday-a		3.86×10^8	8.0×10^8	6.25×10^7	7.4×10^8
Thursday-b		7.72×10^8	1.0×10^9	1.25×10^8	1.0×10^9
Thursday-a		3.55×10^8	8.0×10^8	3.12×10^7	7.4×10^8
Friday-b		7.10×10^8	1.0×10^9	6.24×10^7	1.0×10^9
Friday-a		3.26×10^8	8.0×10^8	1.56×10^7	7.4×10^8
Saturday-a		6.52×10^8	1.0×10^9	3.12×10^7	1.0×10^9
Sunday-a		1.30×10^9	1.0×10^9	6.24×10^7	1.0×10^9
Monday-a		2.60×10^9	1.0×10^9	1.25×10^8	1.0×10^9

*Assumptions: (*1*) no hypoxic fraction, 10^9 initial cell population; (*2*) tumor doubling time = tumor generation time = 24 hrs; (*3*) factors derived from the curves for vascular endothelium and epidermoid cancer cells for 200 rads shown in fig 2; and (*4*) b = before treatment, a = after treatment.

TABLE 3.—One week of X-ray therapy: 200 rads for 5 days, no proliferation with and without repair*

		Repair active		No repair	
		Cancer cells	Normal cells	Cancer cells	Normal cells
Day	*Factor:*	*0.46*	*0.80*	*0.25*	*0.74*
Monday-b		1.0×10^9	1.0×10^9	1.0×10^9	1.0×10^9
Monday-a		4.6×10^8	8.0×10^8	2.5×10^8	7.4×10^8
Tuesday-b		4.6×10^8	8.0×10^8	2.5×10^8	7.4×10^8
Tuesday-a		2.1×10^8	6.4×10^8	6.2×10^7	5.48×10^8
Wednesday-b		2.1×10^8	6.4×10^8	6.2×10^7	5.48×10^8
Wednesday-a		9.7×10^7	5.12×10^8	1.55×10^7	4.06×10^8
Thursday-b		9.7×10^7	5.12×10^8	1.55×10^7	4.06×10^8
Thursday-a		4.46×10^7	4.10×10^8	3.87×10^6	3.00×10^8
Friday-b		4.46×10^7	4.10×10^8	3.87×10^6	3.0×10^8
Friday-a		2.05×10^7	3.28×10^8	9.67×10^6	2.22×10^8
Saturday-b		2.05×10^7	3.28×10^8	9.67×10^6	2.22×10^8
Sunday-b		2.05×10^7	3.28×10^8	9.67×10^6	2.22×10^8
Monday-b		2.05×10^7	3.28×10^8	9.67×10^6	2.22×10^8

*Assumptions: (*1*) no hypoxic fraction, 10^9 initial cell population; (*2*) both tumor and normal cell fractions are division-blocked; (*3*) factors derived from fig 2; (*4*) b = before treatment, a = after treatment.

An important implication of this type of analysis is the limitation of normal cells for repopulation compared to that of tumor cells when both repair and proliferation take place. Cure is prevented by tumor repopulation alone. In combined drug/X-ray therapy protocols, repopulation should be allowed to take place only to the extent that the normal tissues have been reconstituted. Inhibition of repair, on the other hand, is beneficial during the entire treatment series only if the tumor cells are the more radiosensitive cell population. This type of analysis suggests that the fundamental goals for applied radiobiologic research in the immediate future should be: (*a*) to develop drugs that are capable of blocking the repair of sublethal X-ray damage; (*b*) to determine as precisely as possible the effects of cycle-active drugs on the repopulation kinetics of irradiated normal tissues in the anatomic areas of relevance to the (potentially) radiocurable cancers; and (*c*) to discover drugs which can even slightly preferentially reduce the shoulder (D_q) of specific tumor types. Existing clinical data indicate that a wide variety of early cancers can be cured by X-ray therapy; this implies that rational exploitation of even minor differences in tumor- versus normal-tissue radiosensitivity (which we frequently call "palliation") may lead to increased permanent control rates if the two parameters of repair and repopulation are correctly manipulated. It should be obvious that the most likely means by which such manipulations can be brought about is through the development of appropriate regimens combining X-ray therapy with chemotherapy, and it is, therefore, of interest to review the current status of drug/X-ray interactions.

Blocking X-Ray Repair Mechanisms With Drugs

The clinical data on X-ray and drug combination studies have recently been reviewed (13,14). Suffice it to say that there exists in the literature no study which unequivocally demonstrates an increased cure rate when a drug and X-ray have been combined. However, it is clear that certain types of drugs can block the repair of sublethal X-ray damage while others cannot. One of the original studies by Elkind et al illustrated that actinomycin D (NSC-3053) could block split-dose repair while inhibitors of protein or DNA synthesis could not (15). Unfortunately, the mechanism for this capacity is still not agreed upon nor is there concrete clinical evidence that actinomycin D has successfully increased the cure rate in any cancer receiving combined therapy, although the results with Wilms' tumor are suggestive (16). But it is well established that radiosensitization can be demonstrated in vitro and that this effect is not limited to actinomycin D. Our own studies suggest that adriamycin (NSC-123127) may be even more useful in this regard as illustrated by its ability to reduce the shoulder and increase the slope of the survival curve for malignant human epidermoid cells in culture, using concentrations well below those attainable in vivo (fig 3). Simple data such as these clearly indicate that radiosensitization of oxygenated cells is possible and invite clinical exploration.

Molecular Basis for Cellular Radiosensitization in Oxygenated Cells

The demonstration that actinomycin D, adriamycin, etc, could block the repair of sublethal damage together with the general acceptance that disruption of DNA integrity is the most probable lethal event in X-ray therapy cell killing have led to a variety of studies on DNA molecular repair mechanisms, particularly since it is well known that actinomycin D binds to cellular DNA. DNA molecular repair mechanisms fall into two general categories, *repair replication* and *DNA strand-break rejoining*, which together comprise the "cut and patch" repair mechanism (17). It should be noted at the outset that there is as yet no universal agreement as to the relationship between either of these repair phenomena and the cellular repair of sublethal damage.

Repair replication.—DNA synthesis occurring as a result of radiation exposure to either ultraviolet light or X-rays was first demonstrated in mammalian cells by Rasmussen and Painter (18). When observed autoradiographically this phenomenon is termed "unscheduled" DNA synthesis; when studied more rigorously using DNA sedimented in CsCl density gradients the phenomenon is generally

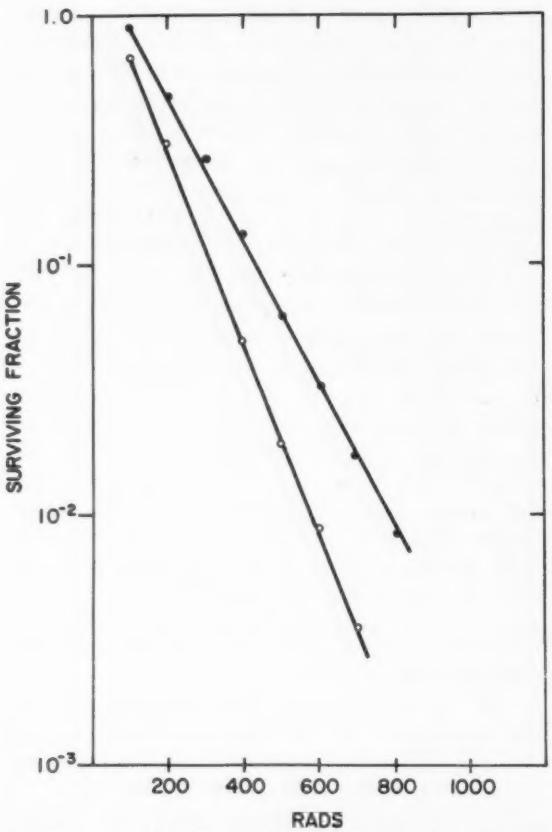

FIGURE 3.—Radiation survival curves for cells with and without adriamycin. Cervix epidermoid cancer cells were irradiated in the presence or absence of adriamycin (0.003 μg/ml) and their survival was followed. The increased slope in the drug-treated culture indicates radiosensitization (open circles = adriamycin).

termed "repair replication" (19). Repair replication as such was first demonstrated in mammalian cells by Painter and Cleaver using high (100 k-rad) X-ray doses (20). Both unscheduled DNA synthesis and repair replication have now been demonstrated in a wide variety of cell types and are now known to involve all four bases and to have variable enzymatic pathways depending on the nature of the molecular damage (21). In our own laboratory we have been interested in the quantitative aspects of repair replication in murine L1210 leukemia cells because of the wealth of pharmacokinetic data already available for the L1210 system (22). L1210 cells are quite active in repair replication (23) and the process can be studied relatively easily by blocking normal DNA synthesis with hydroxyurea or cytosine arabinoside,[3] neither of which affects repair replication.[4] In L1210 cells the process is very poorly dose dependent (like other lines) and requires about 3 hours to reach completion after a 14-k-rad dose. We have been able to demonstrate repair replication at doses of 1000 rads and less using the cytosine arabinoside block method.[4] Similarly, Brent and Wheatley could show repair replication after 500 rads by employing synchronized G_1 cells, and they further showed that the repaired DNA replicated normally (24). There is now, therefore, no reason to doubt that repair replication occurs after clinical X-ray doses where cell survival is significant. The kinetics of repair replication more closely follow those of Elkind-Sutton repair than does strand-break rejoining (*see* below). Moreover, it has been shown that X-ray-induced repair replication is inhibited by actinomycin D (23) and by other DNA-binding antibiotics such as adriamycin and daunorubicin.[4,5] Based on these results one might propose repair replication as a prime candidate for the molecular basis of sublethal damage repair. Unfortunately, there exists a marsupial cell line which shows essentially no repair replication after X-ray exposure (25), although repair replication does occur after ultraviolet ray exposure (26). Since the marsupial line has a shouldered survival curve and shows Elkind-Sutton repair, this observation casts doubt on the role of repair replication in the cellular repair of sublethal X-ray damage. Moreover, we have now isolated two lines of human cancer cells from different patients with cancer of the cervix, neither of which shows repair replication by the standard assay (fig 4); however, these lines are quite radiosensitive with D_qs less than 100 rads and it is

[3] Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1. Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

[4] Lee YC, Byfield JE, Bennett LR, et al. Radiation repair in L1210 leukemia cells: repair replication. Submitted to *Cancer Research*.

[5] Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin.

questionable as to whether they show Elkind-Sutton repair. Because of these difficulties, the role of repair replication in the repair of sublethal damage remains problematic. It should be emphasized, however, that repair replication in its various forms may be quite important in long term genetic damage. In addition, its inhibition by drugs in cells in which it *is* operative may be damaging despite its absence in other cell lines, since faulty repair (for example, excision with incomplete or inaccurate repair) may be worse than no excision at all, particularly where redundant DNA is involved.

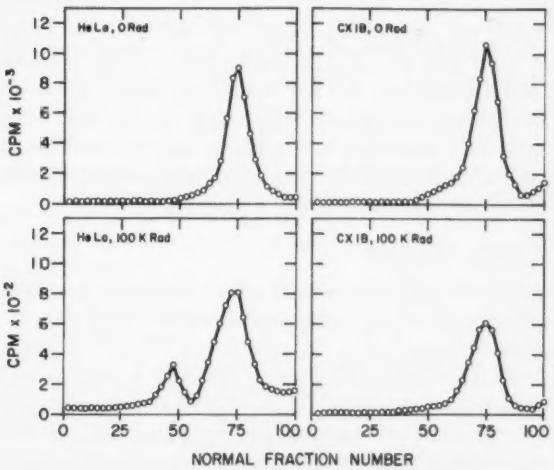

FIGURE 4.—Example of repair replication in mammalian cells. HeLa cells were grown for one doubling time in bromodeoxyriboside (BUdR) and then irradiated (100 k-rad) and labeled with ^{3}H-thymidine for 1 hr. Control cells received the same treatment but no radiation. After labeling, the cells were harvested, the DNA was extracted, and the cells were subjected to alkaline CsCl gradient centrifugation analysis. The control cells show a single radioactive peak to the right of the gradient indicating label uptake in only new DNA (semiconservative synthesis), while the radiated cells show reduced uptake in the right-hand peak and uptake into a second (heavy) peak indicating reduced normal DNA synthesis (righthand peak) and repair replication (lefthand peak) which could be obtained only by substitution of new bases into the preexisting (heavy) BUdR-containing DNA strands (*see* text and ref 19). In an identical set of experiments, a recently isolated cervix cancer cell line (CX 1B) was similarly studied but no repair replication could be identified.

DNA strand-break repair.—The introduction of alkaline sucrose gradient methods in 1966 (27) has permitted a variety of studies on the reparability of mammalian DNA single-strand breaks (28) and, under neutral gradient conditions, a more limited analysis of double-strand breaks. Since chromosome damage is probably related to, or responsible for, most of the cell killing that follows low-dose radiation, unrepaired strand breaks have particular relevance (29). Bacterial strains quite sensitive to radiation commonly show impaired or delayed strand-break rejoining (30) and this can be affected by chemicals (31). Unfortunately, the reparability of strand breaks in mammalian cells cannot be readily distinguished from other forms of repair (in aggregate called repair replication) because only a minority of X-ray-induced breaks present a suitable substrate for the rejoining enzyme, polynucleotide ligase (32,33). In addition, there are now a variety of gradient techniques available which give differing results when different parameters are manipulated. From the standpoint of attempting to relate Elkind-Sutton repair to decreasing numbers of residual breaks there is substantial evidence to suggest that no relationship exists (34,35). For example, in most mammalian cells incubated at 37 C after irradiation, the rejoining of single-strand breaks is essentially complete at 30 minutes when Elkind-Sutton repair is only partially complete (28). However, the radiation utilized in most such studies to elicit single-strand breaks involved doses leaving negligible surviving cells, and it is not certain that the gradient shifts studied accurately reflect the same distribution of chemical lesions that occur after low-dose irradiation. A major area of interest, therefore, continues to be a comparison of the effect relevant variables have on Elkind-Sutton repair and strand-break rejoining. In their initial studies on the effect of metabolic inhibitors on split-dose repair, Elkind and his colleagues noted that of the inhibitors studied only actinomycin D blocked the repair of sublethal damage (15). They interpreted this effect as being due to the drug's disturbance of the microchemical structure of the radiated chromatin, largely because actino-

mycin D had already been reported not to inhibit single strand-break repair because split-dose repair was only poorly temperature-dependent and not inhibited by hypoxia. Presumably, therefore, a nonenzymatic process was involved in cellular split-dose repair (15). However, it has recently been shown that complete hypoxia (which is difficult to obtain without special culture conditions) *does* completely block split-dose repair (36), and it is also now known that the rejoining of the simplest strand breaks occurs even at substantially reduced temperatures (37). In addition, the studies which initially reported no inhibition of single strand-break rejoining by actinomycin D required relatively high X-ray doses (5 k-rads or greater) and rejoining was substantially incomplete in both control and drug-treated cultures (34,35). If a gradient procedure is chosen which is capable of demonstrating the repair of DNA strand breaks after low X-ray doses, it can be shown that very low concentrations of actinomycin D inhibit this rejoining phenomenon (37). However, the same gradient methods also reveal that low concentrations of actinomycin D can either induce additional strand breaks or cause unravelling of some residual double-strand regions, making precise interpretation impossible (38). Other DNA-binding antibiotics have similar properties suggesting that radiosensitization (by inhibition of repair mechanisms) is intimately related to DNA/drug-binding potential.[6] This hypothesis is supported by negative studies on antimetabolites which do not show any effect on break rejoining (37). Moreover, the studies described above clearly have shown that DNA-binding drugs inhibit X-ray-induced repair replication (23) and both X-ray-induced unscheduled DNA synthesis (39) and ultraviolet-induced repair replication (40). Since repair replication assays measure base insertion it seems likely that repair replication inhibition would necessarily leave gaps in the DNA strands unless the antibiotics affected only the excision steps. Since such gaps are not detected by the standard 5%–20% alkaline sucrose methods (34,35) but may be detected by the low-dose procedures (37), it remains plausible to postulate that idiosyncracies of the gradient methods may not allow a study of all the existing strand breaks and that the DNA-binding antibiotics can both inhibit strand-break repair and break strands. Alternatively, the original interpretation of Elkind et al (15) may be correct, namely, that the antibiotic effects are exerted at the level of a quaternary structure or "complex" as it has been termed (41). From the practical standpoint it would appear more useful to ignore these discrepancies for the time being and to concentrate our efforts on the development of more of these kinds of DNA-binding antibiotics since they clearly meet the best criteria of radiosensitization, an increased slope on the cell survival curves (fig 3). In addition, these drugs tend to show predilections for specific tissues (such as bleomycin [NSC-125066] for epithelial cells and adriamycin for cardiac tissue), and such differential toxicity has long been a major aim in cancer chemotherapy screening.

CONCLUSIONS

From this discussion it appears reasonable to suggest that appropriate combinations of chemotherapeutic agents and X-ray therapy may allow greater control rates in a significant number of cancers. At least two different types of agents will probably be required: cycle-active drugs to block repopulation and inhibitors of radiation repair mechanisms, perhaps initially supplemented by sensitizers of hypoxic cells (1). As illustrated in tables 2 and 3, it will be just as critical to utilize correct regimens as it will be to select the right drugs. As yet, little is known concerning the optimal pharmacokinetics of such agents when given in the context of fractionated radiation. Moreover, the tolerance of various normal tissues must be determined for each combination with reference to individual anatomic areas and to the overall implications of radiation necrosis in individual organs. Such toxicity studies are difficult, expensive, and complicated by the volume effect in the induction of radionecrosis which render small animal studies of questionable relevance. Yet the number of patients dying due to local-treatment failure is large,

[6] Byfield JE, and Lee YC. Radiation repair in L1210 leukemia cells: DNA single strand break rejoining. Submitted to *Cancer Research*.

and the existence of significant X-ray control rates already implies that the problem is fundamentally one of cell number rather than tumor-cell biology. Finally, in contrast to chemotherapy for disseminated cancers, the therapist has the advantage that systemic toxicity will probably be less because a significant part of the therapeutic insult, the X-ray treatments, are directed locally. Clinical studies designed to test these hypotheses are now in progress. Perhaps, armed with the weapons of modern pharmacokinetics, we can begin to apply the knowledge that basic radiobiology has given us.

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X-Ray Sensitization by Halopyrimidines [1]

Waclaw Szybalski [2,3]

SUMMARY

The principal subjects of this review are the sensitization of cells toward ionizing radiation by incorporation of the halogenated thymidine analogs into their DNA, and the application of this principle to the radiotherapy of the neoplastic diseases. The analogs include 5-chloro-, 5-bromo-, and 5-iodo-deoxyuridine; 5-bromo- and 5-iodo-deoxycytidine; and trifluoromethyldeoxyuridine (trifluorothymidine). 5-Fluorodeoxyuridine, a deoxyuridine analog, is not incorporated into DNA (with the exception of the uracil-containing DNA of phage PBS2) but promotes the incorporation of the halogenated thymidine analogs by blocking the de novo synthesis of thymidylic acid. Over half of the thymidine in the cells' DNA can be replaced by the analogs, rendering the cells up to four times more sensitive to X-rays. In clinical application, selective incorporation of the analogs into the tumor DNA is hampered by several factors, including enzymatic cleavage, dehalogenation, rapid elimination, toxicity, and incorporation into normal replicating tissues. However, in several clinical trials employing intra-arterial infusion directed toward brain tumors, impressive radiotherapeutic gains were reported. Several approaches for overcoming the problems encountered in the clinical application of radiosensitization by the halogenated thymidine analogs are discussed. The physical and chemical mechanisms underlying the radiosensitization process are briefly outlined.

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It was only with misgivings that I accepted Dr. Hall's invitation to address this Symposium, since I am not currently active in the field of radiosensitization. My present research interests are centered around the transcriptional controls of gene expression (1). As to the subject of this session on the clinical applications of radiosensitization, my preliminary assessment could be summed up in these words: "a good idea which failed." However, after reviewing the present status of the field I became more optimistic, since it now appears that this principle might be useful after all, at

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[2] McArdle Laboratory for Cancer Research, University of Wisconsin, Madison.

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least in particular forms of neoplastic disease.

Let me first summarize the idea and the rationale behind it. The principle was a *selective* sensitization of neoplastic cells to X-rays by incorporation of 5-halodeoxyuridines into their DNA. The following considerations supposedly made this approach selective and effective:

1. Halogenated thymidine analogs are incorporated only into actively dividing cells during DNA replication.
2. Neoplastic cells are actively proliferating, usually more so than normal tissues.
3. Cells in which the thymidine residues of DNA have been replaced by 5-halodeoxyuridines become several times more sensitive to the lethal effects of X-rays.

Since radiotherapy already plays an important clinical role in the management of cancer, a major and selective increase in its efficacy should be a significant advance. This rationale was outlined at several meetings during 1959–1961 (2–8) and was included in our first comprehensive paper on radiosensitization of human cells published in 1960 (9).

It might be of historical interest to outline briefly my personal recollections of how this idea originated, especially as related to the radiotherapy of tumors and to the contributions of our laboratory. During the period of 1956–1958, we became interested in the mechanisms of mutagenesis in both bacterial and human cells (10). In our studies on the mutagenic effects of radiation we happened to employ 5-bromodeoxyuridine (BUdR) and 5-iododeoxyuridine (IUdR) as co-mutagens and as density labels for DNA. To our surprise, we found that replacement of thymidine by the halogenated analogs renders the cells extremely sensitive to X-rays and ultraviolet (UV) light. Since human cells as well as bacteria were affected, this accidental observation immediately suggested to me that the 5-halodeoxyuridines might serve as sensitizing agents in the radiotherapy of tumors. To explore the clinical possibilities, I established in 1958 a liaison with the Radiotherapy Section of the Middlesex Hospital in New Brunswick, New Jersey. The systematic evaluation of the UV and X-ray sensitivity of human cells grown in vitro became the subject of the doctoral thesis of Boshko Djordjevic (11), who had just joined our laboratory at the Institute of Microbiology of Rutgers University. At the same time we became aware that the phenomenon of UV light sensitization by 5-bromouracil had been discovered by Greer and Zamenhof and presented at the 1957 meeting of the American Chemical Society (12). Therefore, we christened the photosensitization phenomenon the "Greer-Zamenhof effect." A more complete account of Greer's work was published in 1960 (13).

Anticipating that radiosensitization by 5-halodeoxyuridines would become an important tool in the newly arising discipline of molecular biology, we began to apply it in various ways. Dr. A. W. Kozinski used BUdR incorporation as a label for the parental DNA in a study of the mode of DNA replication of the bacterial virus ϕX-174 (14). Mrs. Z. Opara-Kubinska and Dr. Z. Lorkiewicz studied the sensitivity of transforming DNA labeled with 5-halodeoxyuridines and, using this tool, established that DNA is the principal target of the lethal X-ray and UV effects (7,8,15). Our early studies on the combined mutagenic effects of radiation and BUdR or IUdR, which included the original observation of the radiosensitization phenomenon, were also finally published (16). At that time we also described a novel method for attaining higher incorporation of 5-halodeoxyuridines into DNA, ie, inhibiting de novo synthesis of thymidylic acid with 5-fluorodeoxyuridine (FUdR), a drug developed by Dr. C. Heidelberger (17). We subsequently applied this principle for labeling and radiosensitizing of human cells (9). Our main efforts became directed toward the study of the X-ray sensitization of human cells, which, after my move to the University of Wisconsin in 1960, became the subject of several papers and the doctoral thesis of Raymond Erikson (18–21). In parallel, Dr. G. Ragni studied the sensitization of human cells by 5-chlorodeoxyuridine (CUdR), BUdR, and IUdR incorporation to inactivation by the decay of incorporated radiophosphorus (22).

The concept of using the halogenated thymidine analogs as radiosensitizers of tumors had some impact on the field of radiotherapy.

Dr. Henry Kaplan, of the Department of Radiology at Stanford University, telephoned me early in 1959 (23) after reading my note in an informal publication (24) and told me that our lead looked promising enough to warrant a major effort on the part of his Department. He organized an informal Conference on Chemical Radiosensitization in the spring of 1960 at Carmel, California, and graciously invited me to participate (5). At the University of Wisconsin I established a close cooperation with Drs. K. H. Clifton, H. Vermund, and F. F. Gollin of the Radiology Department, Drs. F. J. Ansfield and A. R. Curreri of the Cancer Research Hospital, and Dr. C. Heidelberger, my colleague at the McArdle Laboratory, which eventually led to a joint publication (25).

Let me now summarize the experiments performed in several laboratories, including ours, which demonstrated the incorporation of halogenated thymidine analogs into the DNA of human cells in vitro and in vivo, as related to X-ray sensitization. The chemical structures of four thymidine analogs, CUdR, BUdR, IUdR, and trifluoromethyldeoxyuridine or trifluorothymidine (F_3TdR), are shown in figure 1. Stereochemical considerations suggest that BUdR is the best thymidine analog since the van der Waals radius of bromine (1.95 Å) is very close to that of the methyl group (2.0 Å). The fifth halogenated pyrimidine, FUdR, is an analog of deoxyuridine since the radius of fluorine (1.35 Å) is much closer to the radius of hydrogen (1.2 Å) in uracil than of the methyl group (2.0 Å) in thymidine. It was possible to obtain quite an efficient incorporation of CUdR, BUdR, and IUdR into the DNA of human cells with a thymidine replacement of as high as 50% (18–22), especially when de novo synthesis of thymidylic acid was blocked by FUdR (fig 2). Figure 3 represents the X-ray survival curves for human cells labeled for 4 days with 2×10^{-6} M analogs (+FUdR). The radiosensitizing effect of increasing concentrations of IUdR is shown in figure 4. The thymidine replacement was higher with BUdR (up to 43%) than with IUdR (24%), but the corresponding X-ray sensitization for IUdR (3.5-fold) was more pronounced than for BUdR (2.9-fold) (20). Similar results were obtained with the cytidine analogs, 5-bromodeoxycytidine (BCdR) and 5-iododeoxycytidine (ICdR), which replace thymidine in DNA, since they are easily deaminated to BUdR and IUdR (20). The sensitization factors for UV light and X-rays, measured as a function of the percent of thymidine replacement, are shown in figure 5. The sensitization with F_3TdR was less impressive and the DNA incorporation was low because it is not a good analog of thymidine (figs 1 and 6; ref 26). In one special case, namely that of phage PBS-2, the DNA of which contains uracil instead of thymine, Lozeron and Szybalski (27) were able to incorporate FUdR into DNA with a resulting small radiosensitizing effect. The possible radiosensitizing effects of FUdR, under conditions when it inhibits its DNA synthesis but is not incorporated into DNA (17,43,53,57), is outside the scope of this review.

Because these results and those of others (29–39) showed that it is possible to attain a very substantial radiosensitization of human cells in vitro, the extension of these findings to the radiosensitization of tumors looked feasible to us. The next obvious step was to attain a similar degree of 5-halodeoxyuridine incorporation into the DNA of spontaneous or transplanted animal tumors and to evaluate

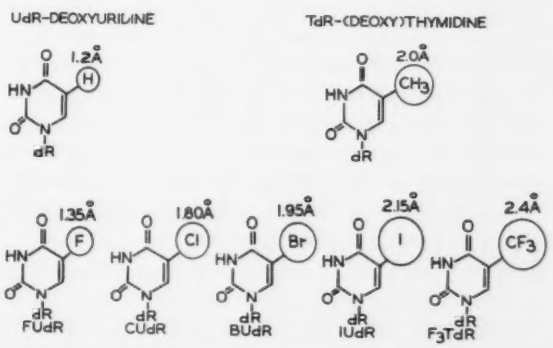

FIGURE 1.—Structures of 2 natural pyrimidine deoxynucleosides, deoxyuridine and thymidine, and their halogenated analogs: FUdR (a deoxyuridine analog) and the thymidine analogs (CUdR, BUdR, IUdR, and F_3TdR). The circles at position 5 represent the comparative sizes of the substituting groups, and the van der Waals radii are indicated above each circle. (*See* refs 7, 17, 87, 264, 273, and 296.)

the X-ray sensitization. Here, however, the *simple* extrapolation from cell cultures to animals failed miserably. Several major problems developed, including (*a*) extensive degradation of the TdR analogs, mainly in the liver, and their rapid excretion (40,41); (*b*) the toxicity of the analogs, especially IUdR (25); and (*c*) incorporation of the analogs into normal nonmalignant but rapidly proliferating tissues, eg, bucal mucosa and other squamous epithelial tissues (25,41). Despite these problems, however, it was possible to attain up to 14% thymidine replacement by its brominated analog in ascites lymphoma cells after intraperitoneal administration to mice of BCdR combined with FUdR (42). Although up to 2.4-fold X-ray sensitization of ascites cells was attained (42), this case is obviously quite special and probably not representative of neoplasms in general, especially solid tumors. A substantial radiosensitization of Sarcoma 180 and ascitic leukemia in mice by IUdR was reported by Heidelberger (5) and by Berry and Andrews (28, 150).

The difficulties encountered with the administration of the 5-halodeoxyuridines suggested several experimental countermeasures.

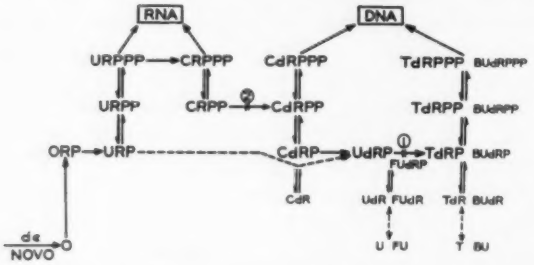

FIGURE 2.—A simplified metabolic map of the synthetic and catabolic pathways for pyrimidine nucleotides. The inhibition of thymidylic acid synthesis by 5-fluorodeoxyuridylate (FUdRP) (circle 1) and the feedback inhibition by several nucleotides of the conversion from nucleotide to deoxynucleotide (circle 2) are indicated. (*See* ref 7.)

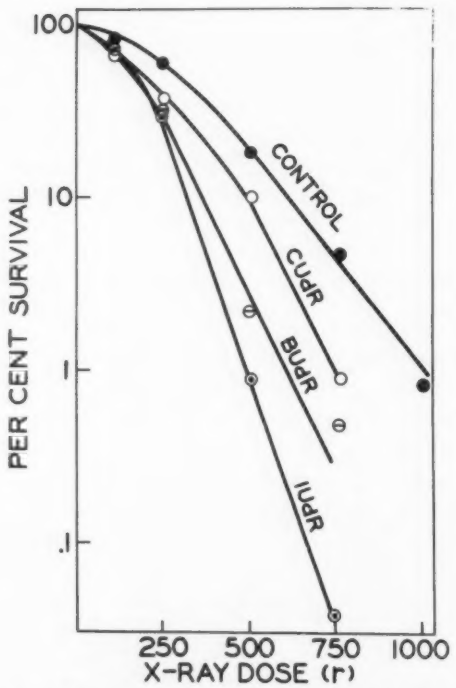

FIGURE 3.—X-ray survival of the human bone marrow cell line D98/AG grown for 4 days prior to irradiation in the absence (control) or in the presence of 0.004 μg FUdR/ml and 2×10^{-6} M CUdR, BUdR, or IUdR. (*See* ref 18.)

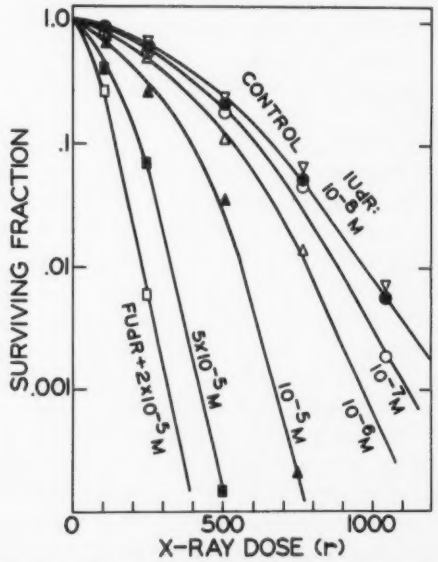

FIGURE 4.—X-ray survival of D98/AG human cells grown for 5 days prior to irradiation in the absence (control) or in the presence of the indicated concentrations of IUdR. The FUdR concentration (steepest curve only) was 10^{-7} M. (*See* ref 19 for other experimental details, including the extent of IUdR incorporation and the associated plating efficiency of the labeled cells.)

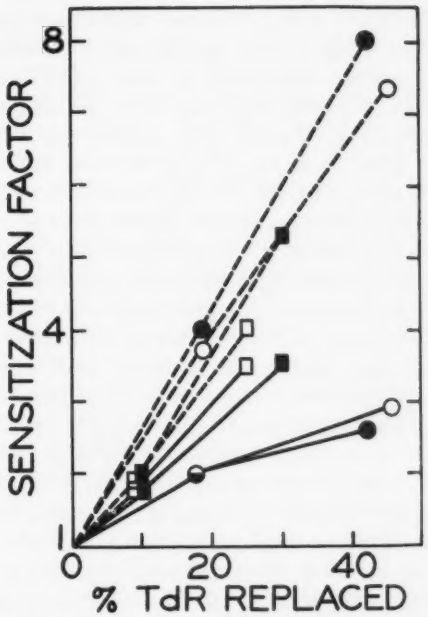

FIGURE 5.—Sensitization factor, calculated as D_w (control)/D_w (labeled), for D98/AG human cells exposed to UV light (dashed lines) or X-ray (solid lines) as a function of thymine replacement by 5-bromouracil (circles) or 5-iodouracil (squares). The cells were exposed to the halogenated deoxyuridine (open symbols) or deoxycytidine (solid symbols) analogs. (*See* ref 20.)

(*a*) Since dehalogenation is most extensive in the liver, several alternative routes of drug administration were proposed, including intra-arterial infusion to the artery feeding the neoplasm, intravenous (iv) administration in the case of early pulmonary metastases, and shunting the hepatic circulation (5,43). Another way to counteract dehalogenation would be to modify chemically the analogs or to use them in conjunction with other agents which would block dehalogenation. The administration of analogs in the depot form (25) should slow the excretion rate.

(*b*) To overcome toxicity one might attempt to administer thymidine to protect the normal tissues, while directing the halogenated analog only to the neoplasm (5). If the analog were injected directly into the artery feeding the tumor, hepatic cleavage and dehalogenation of the drug would actually provide the positive effect of detoxification (43).

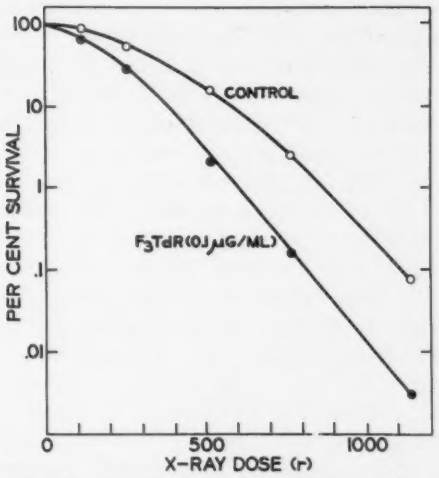

FIGURE 6.—X-ray survival of D98S human cells grown for 2 days prior to irradiation in the absence (control) or in the presence of 0.1 μg F_3TdR/ml. Cell survival in unirradiated controls was 60%. (*See* ref 26.)

(*c*) To minimize analog incorporation into normal tissues it is necessary to select types of neoplasms that are surrounded by nonproliferating tissues (5,43). Several other measures already discussed could also be helpful in this respect.

It is obvious from the above discussion that the clinical use of halogenated deoxyuridines as radiosensitizers would require a discriminating selection of tumors most suitable for such combined therapy, as well as special methods of drug administration and countermeasures to decrease degradation, rapid elimination, and toxicity. One of the earliest clinical radiosensitization attempts using BUdR was that of our Symposium chairman, Dr. T. C. Hall (278).

To illustrate the problems outlined above, I shall refer to a few selected attempts to apply the 5-halodeoxyuridines to the radiotherapy of tumors (43). Bagshaw et al (44) at Stanford Medical School initiated a randomized clinical study to evaluate the radiosensitizing effects of BUdR administered intra-arterially in patients with advanced carcinoma of the head and neck. BUdR was administered by daily infusions of up to 1 g of BUdR per day for as long as 30 days. Irradiation was at the rate of 200 rads/day, up to a total of 6000 rads.

Several regimens of drug administration and split-course radiotherapy were evaluated. The incorporation of BUdR into tumor DNA was substantial and in one case reached almost 5% thymidine replacement. Radiosensitization also was striking, but unfortunately was directed also toward adjacent normal tissues. For example, when BUdR was administered to only one side of the tongue while both sides were irradiated, a striking increase in the tissue reaction was observed on the side receiving BUdR. Despite the radiosensitizing effect, no therapeutic gain in the patients receiving both BUdR and radiation was observed when compared with the group treated with radiation alone (43,44). In retrospect, it appears that the choice of carcinoma of the head and neck was unfortunate because of the rapid turnover of the bucal mucosa and consequent incorporation of the analog into the neighboring normal oral tissues.

In contrast to this discouraging result, Hoshino et al (45–47) and Sano et al (48–52) reported a considerable therapeutic gain for brain tumors treated with the combination of BUdR and X-rays. They recognized that in the central nervous system rapidly growing tumors would be the only replicating tissue, since DNA synthesis is very low in endothelial or glial cells and virtually nonexistent in neural tissue. Thus, they avoided the disadvantage of rapidly proliferating bucal mucosa encountered in the experiments of Bagshaw et al (43,44) or Fletcher et al (53), and in analogous, but apparently more successful, studies reported by Tanaka and Takahashi (54,55). Moreover, (*a*) brain tumors are solitary and rarely metastasize; (*b*) normal brain tissue is usually protected from administered chemicals by the blood-brain barrier, whereas tumors are exposed because of the breakdown of this barrier; and (*c*) intra-arterial or intrathecal infusion is relatively easy (51). The treatment (denoted BAR therapy; ref 50) consisted of daily infusion of 0.6–1 g of BUdR per day for 4–7 weeks with a total dose of 25–45 g, and a daily radiation dose of 150–200 rads up to a total of 6000 rads. The results obtained with 107 cases were rather impressive. For moderately or highly malignant gliomas the 1-year survival rates after BAR therapy were 88% or 68% respectively, whereas survival for the latter after surgery alone was about 5%. At 2 years after treatment, the corresponding survival values were 72% or 44% (BAR) and 0, and at 3 years, 54% or 18% (BAR) and 0. In summary, hardly any patients survived 1 year after surgery alone, whereas 44%–76% were protected for 2 years and 18%–54% for 3 years after combined BAR therapy. Moreover, Sano et al (51) reported that the "quality" of life for half of the surviving patients was good to excellent and that they could "lead a useful life." They are very optimistic about improving the BAR therapy and obtaining still higher survival rates. Commenting on these studies, Doggett and Bagshaw (43) conclude that these results are "impressive indeed," especially considering the lethal nature of the treated neoplasms. However, they stress that no randomized control group of patients treated with radiation alone was included, and that in the case of the surgical controls, in contrast to US practice, the skull flaps are not replaced in Japanese patients after the craniotomy, which might contribute to a longer survival because less intercranial pressure develops during regrowth of the neoplasm. The 1-, 2-, and 3-year survival rates after surgery combined with radiation alone were cited (51) to be 40%, 20%, and 16% for the gliomas. Clinical gains for the combined BAR therapy of brain tumors were reported also by Mundiger et al (56). Some gains in survival were obtained by Gollin et al (57) for the combined IUdR and X-ray therapy of inoperable squamous cell carcinoma or adenocarcinoma, as compared with X-ray alone. An impressive response and over 5-year survival for inoperable fibrosarcoma of the femur treated with intra-arterial infusion of IUdR combined with X-ray therapy were observed by F. J. Ansfield (personal communication).

Promising results were also obtained at Stanford University, where Brown et al (58) and Goffinet et al (59) have developed an experimental model system for performing prolonged intra-arterial infusion in the mouse. Using BCdR, they obtained an average dose-modifying factor (DMF) of about 1.3 (high-

est value, 1.5) for transplanted sarcomas and a somewhat lower one for gliomas, without significantly increasing the skin reaction. Moreover, for "micrometastases" in the lungs, J. M. Brown (personal communication) has obtained about 1.5-fold sensitization, as judged from the slope ratio of the X-ray survival curves. According to Doggett and Bagshaw (43), these experiments show a definite therapeutic gain equivalent to delivery of about 30% more radiation to the sarcomas (or 12% to the gliomas) with no increase in damage to the normal cutaneous tissues. Also, the early pulmonary metastases might be ideally suited for clinical radiosensitization therapy, because (*a*) the replication of normal cells in the lung is probably minimal whereas early metastases would replicate rapidly and thus selectively incorporate BUdR, BCdR, or other halogenated pyrimidines; (*b*) a substantial blood supply is via the pulmonary arteries, which would permit introduction of sensitizer to the pulmonary circulation by simple iv injections; and (*c*) since the lung is quite radiosensitive, the selective sensitization of tumors is of great practical importance. Doggett and Bagshaw (43) predict that a DMF of 2–3 might be achieved with BUdR, which could lead to destruction of early metastases within the limits of tolerance of pulmonary tissue.

As already alluded to, prevention of the degradation of the deoxynucleoside to the free base, its dehalogenation, and other kinds of enzymatic inactivation of 5-halodeoxyuridines would enable more successful clinical application of these radiosensitizers. Their in vivo degradation was noted in several studies (40, 60–64), some even preceding the proposal of their use as radiosensitizers (60). One of the first attempts to circumvent the catabolic degradation of the 5-halodeoxyuridines to the free base was by the use of the deoxycytidine analogs, BCdR and ICdR (41,62); it is known that deoxycytidine and its analogs are not substrates for mammalian nucleoside phosphorylases (40,65–67). This approach was unsuccessful, since both BCdR and ICdR were rapidly deaminated in vivo by the cytidine deaminase to BUdR and IUdR and thus did not offer much advantage over the use of the latter two analogs (41). Moreover, the 5-halodeoxycytidines are very poor substrates for the deoxycytidine kinase, and if not deaminated cannot be successfully phosphorylated to their corresponding deoxynucleotides prior to their incorporation into DNA (68,69). Extensive studies on the prevention of the catabolic in vivo degradation of the thymidine analogs were carried out by Kriss and his colleagues (40, 41,70–72), by Langen et al (73–75), and more recently in the laboratory of Dr. S. Greer (68, 69,76–79). The inhibitors of dehalogenation include 5-diazouracil and 5-cyanouracil (76–78), whereas 5-diazouracil blocks this and other catabolic pathways, and various purine deoxynucleosides act as a source of deoxyribose-1-phosphate to convert halogenated pyrimidines to deoxynucleosides (73,76,79,80). Tetrahydrouridine inhibits the deamination of cytidine (68). Deoxyglucosylthymine inhibits uridine-deoxyuridine phosphorylase, and the 6-amino derivatives of thymine, bromouracil, and iodouracil block thymidine phosphorylase (74,75). It is hoped that some of these and other analogous inhibitors will act as effective adjuncts in improving both the radiosensitizing and antiviral effects of the halogenated thymidine analogs.

Another approach to increasing incorporation of the thymidine analog, namely IUdR, was its use in depot form as a suspension of microcrystals in an aqueous gel of aluminum monostearate (25). This mode of administration resulted in 2.3–5.5 times greater incorporation than injection of a saline solution of IUdR. In the same study it was observed that IUdR incorporated into tumors remains stable for some time after drug administration is interrupted, whereas the IUdR level drops rapidly in many normal tissues because of the sloughing and migration of maturing cells (25).

The detailed mechanisms underlying the radiosensitizing effects of the 5-halodeoxyuridines are not known. It is clear, however, that they must be incorporated into DNA, replacing thymidine, and that the sensitization of the cell parallels the sensitization of its DNA component (7,8,15,81). One reason for the difficulties in assessing the exact mechanism of radiosensitization is that substitution of the halogen atom for the methyl group of thymine results in a whole gamut of effects, including

primary and secondary radiochemical events and also interference with the repair mechanisms. Thus, the radiosensitizing effects depend on the conditions of irradiation and the kinds of repair mechanisms active in the particular type of cell. The principal mechanism of sensitization, therefore, may vary in different biologic systems.

I am not planning to review here the literature on the mechanisms of sensitization in any detail. Earlier and recent reviews and papers of Freifelder (82), Hagen (83), Hutchinson (84), Myers (85), Setlow and Setlow (86), Szybalski (7,8,81,87), Fielden et al (88), Wacker (89), Ward (90), and Bhatia and Schuler (91) have covered this field. My personal feeling is that for both UV- and X-radiation one of the principal reactions is the abstraction of the halogen from the halogenated analog, leading to the formation of two very reactive groups or radicals: the halogen and the uracilyl residue (89–92). The secondary reactions involving radicals or active groups are the basis of the major radiochemical damage to DNA. One of the simplest and most illustrative experiments showing the great radiolability of the 5-halodeoxyuridines is that described by Wacker et al (92, and unpublished data). If one irradiates a mixture of IUdR and starch, the suspension turns blue, indicating that iodine is released which is detected by the iodine-starch reaction. The conversion of the 5-halouracil to uracil is probably not the most lethal reaction, but many ensuing secondary rearrangements are potentially lethal. The importance of the secondary changes is demonstrated by the experiments of Hotz and collaborators (93–96), who have shown that radical scavengers, eg, cysteamine, can abolish the radiosensitizing effects of the 5-halodeoxyuridines. The desensitization by scavengers appears to lead to the formation of some DNA derivatives which then must be eliminated by the biologic repair mechanisms, since such desensitization is inoperative for single-stranded DNA which cannot be repaired (94). Analogous conclusions were reached by Freifelder (82,97), who irradiated phage under various conditions. The model discussed by Szybalski (87) includes the various modes of DNA irradiation which might or might not lead to sensitization. This model should be amended to include the formation of radicals not only in the solution surrounding the DNA but also in the DNA itself by abstraction of the halogen, as described above.

As already discussed, the halogenated thymidine analogs alone exhibit some toxicity, especially at higher concentrations. This is really not too surprising, and actually one should marvel at how the cell and even the whole organism can tolerate such a profound chemical change in DNA, its most vital component. DNA is not only the template for transcription and its own replication, but is also studded with many recognition sites for specific proteins as signals for the initiation, control, and termination of transcription (eg, promoters, operators, and terminators) or replication (eg, the origin of replication) (1). The interactions between these recognition sites and the corresponding control proteins (and also the restriction nucleases) might be profoundly affected by the incorporation of halogenated analogs into DNA. It is known that replacement of thymine with 5-bromouracil stabilizes the secondary structure of DNA, as indicated by an increase in the melting temperature (98). Furthermore, these changes and the partial inhibition of DNA replication conceivably might lead to the induction of viruses carried in the cell genome (1). It is not surprising, therefore, that these analogs appear to profoundly influence many subtle processes, including cell differentiation and dedifferentiation, in addition to affecting radiation repair and producing generalized symptoms of toxicity.

Since, in tune with the theme of this Symposium, the purpose of my presentation was to demonstrate the potential of radiosensitization by the halogenated thymidine analogs for cancer therapy, I have not included references to most of the papers dealing with photochemical sensitization, especially by UV light. However, several reviews and some early papers dealing with this subject are included in the references (4,7,9,11,13,20,81,84,86,89,114,132,133, 222,228), especially since there is a possibility that with the development of powerful UV-laser techniques, the topical phototherapy of some neoplasms would become feasible. Moreover,

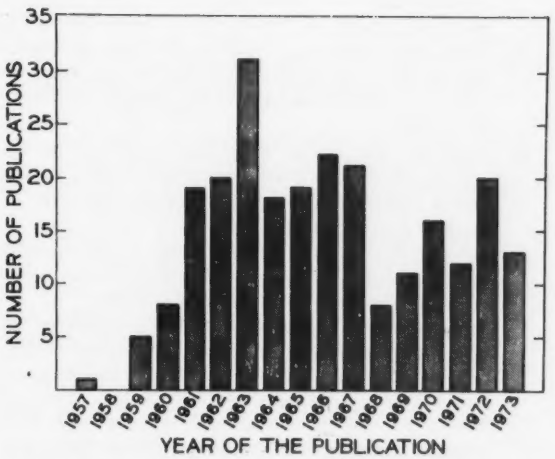

FIGURE 7.—Number of papers, abstracts, and reviews related to radiosensitization by the halogenated thymidine analogs and its clinical applications published during the years 1957–1973. Emphasis is on sensitization to ionizing radiation; the publications devoted only to photosensitization by the halogenated analogs are not included, with the exception of general reviews or books and several early communications published mainly in the years 1957–1961.

I have assembled a rather exhaustive list of references to the books, reviews (83–87,89,90, 99–139), papers, and abstracts which deal with the sensitizing effects of the 5-halodeoxyuridines in conjunction with ionizing radiation (140–258), in addition to those papers already mentioned. The number of communications relating to the latter subject and published during the last 17 years (fig 7) gives some indication as to the active interest in this field. Moreover, Drs. F. J. Ansfield, L. Chwang, G. M. Cooper, M. Fox, U. Hagen, H. S. Kaplan, B. A. Kihlman, M. Klimek, F. K. S. Koo, P. Langen, P. J. Lindop, M. Lion, Z. Lorkiewicz, J. R. Maisin, F. Mundinger, S. Okada, Z. Opara-Kubinska, A. M. Rauth, Y. Tanaka, and K. G. Zimmer have most recently supplied helpful comments and drawn my attention to additional publications related either directly to sensitization by the halogenated analogs (263,268,269, 272,274–278,281–283,285–290), to their administration (292–295), to catabolism and its inhibition (259–262,265–267,270,271,279,280,284, 291), or to their structure (264,273,296).

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Nitroheterocyclic Drugs as Selective Radiosensitizers of Hypoxic Mammalian Cells

J. D. Chapman,[1] A. P. Reuvers,[1] J. Borsa,[1] J. S. Henderson,[2] and R. D. Migliore [2,3]

SUMMARY

The proposal to use chemicals that selectively radiosensitize hypoxic cells in combination with radiotherapy is based on the assumption that hypoxia limits the effectiveness of radiation in the treatment of some solid tumors. The ideal compound for this application would be expected (*a*) to mimic completely the radiosensitizing effectiveness of molecular oxygen at nontoxic concentrations, (*b*) to metabolize slowly, and (c) to diffuse rapidly into the hypoxic regions of tumors after administration. Electron-affinity, a chemical property, has been demonstrated as important in the design of radiosensitizers for hypoxic mammalian cells. The electron-affinity of nitrobenzene is near the threshold required to demonstrate radiosensitizing effectiveness in Chinese hamster cells growing in vitro. Various nitroheterocyclic structures, many of which are in current clinical use as antibacterial and antiprotozoal agents, have been shown to have excellent radiosensitizing properties. Success in applying these cellular sensitizers to animal tumor studies has been limited by the inability to reach the target hypoxic cells with the drugs and hold them in a high enough concentration for the duration of the radiation treatment. Compounds which have been shown effective in animal tumors display a relatively long half-life in the blood ($T\frac{1}{2} \geq 80$ min), a high lipid solubility, and a high water solubility. The nitroheterocycles effect radiosensitization of hypoxic mammalian cells predominantly by adding to the cellular pool of radical-oxidizing species. Thereby they enhance the fixation of neutral radicals generated by the radiation in cellular target molecules with resultant production of lethal forms. This mechanism of action is analogous to that of oxygen and other electron-affinity compounds.

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[1] Medical Biophysics Branch, Whiteshell Nuclear Research Establishment, Atomic Energy of Canada Limited (AECL), Pinawa, Manitoba.

[2] Department of Pathology, Faculty of Medicine, University of Manitoba, Winnipeg, Canada.

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Ionizing radiation has become the agent of choice for the treatment of many human tumors. Current radiotherapy regimens are founded on empirical clinical studies and strive to optimize the growth regression of the tumor treated. Success is limited by the total dose that the normal tissue, adjacent to the tumor, can tolerate. This rarely exceeds 4000–6000 rads over about a 6-week period. Regrowth of the tumor at the primary site is thought to result from more resistant tumor cells which

have survived the lethal action of the most practical radiation treatment. These resistant cells are an important factor in the poor local control achieved with radiation in the case of some tumors.

Large variations in the radiosensitivity of a cell occur as the concentration of oxygen that is present at the time of irradiation is changed. Cells in air-saturated environments are typically three times more sensitive to radiation than cells kept oxygen free. Hypoxia, the state of low oxygen tension, characteristically afflicts a fraction of the cells in solid tumors because by its nature a tumor outgrows the supply of oxygen and nutrients carried to it by the vascular system. Evidence indicates that such hypoxic cells in animal and human tumors are the radioresistant ones which limit radiocurability. Numerous approaches have been suggested to exploit the radiosensitizing effect of oxygen and to enhance cures in clinical radiotherapy. These have been discussed by Duncan (1).

A recent approach under active investigation combines the use of certain chemicals with radiation. The chemicals are those that mimic the radiosensitizing effect of oxygen. Oxygen is metabolized rapidly by tissues. We hoped to discover chemical structures that acted like oxygen in the radiation chemistry of cell inactivation but that were not rapidly metabolized. Such chemicals that could diffuse their radiosensitizing influence into hypoxic regions of tumors could prove useful in radiotherapy. Criteria considered important in selecting such compounds for potential clinical use include:

a. selective radiosensitizing effectiveness against hypoxic cells;

b. nontoxicity at radiosensitizing concentrations;

c. effectiveness against both proliferating and nonproliferating cell populations under various physiologic conditions;

d. resistance to rapid metabolization by tissue; and,

e. diffusibility through tissue and into hypoxic regions of tumors within a short time.

In this symposium we review studies which have led to a large selection of chemical structures effective in selectively radiosensitizing hypoxic cells in vitro and to a better understanding of chemical properties important for delivery of such sensitizers to hypoxic cells in solid animal tumors.

SELECTION OF RADIOSENSITIZING STRUCTURES

Chemicals that radiosensitize cells to the lethal action of ionizing radiation have been studied over the past 25 years. Many such studies and hypotheses concerning the phenomena of chemical radiosensitization have been reviewed recently by Bridges (2) and Adams (3). The use of radiosensitizers in clinical radiotherapy has been reviewed recently by Bleehem (4). Adams has described compounds that radiosensitize hypoxic mammalian cells selectively and show promise of successful clinical application (5). Current interest in this class of radiosensitizers stems from (*a*) the suggestion by Adams and Dewey (6) that the chemical property, electron-affinity (EA), relates radiosensitizing effectiveness to chemical structure, and (*b*) the improved understanding of cellular radiobiology resulting from recent studies of its chemistry (7,8). By early 1970 only two reports had appeared describing the selective radiosensitization of hypoxic mammalian cells. One study used glyoxal derivatives (9). The other study used the stable-free radical, triacetoneamine-*N*-oxyl (10). Numerous EA structures had been shown to radiosensitize hypoxic bacterial cells (11), but attempts made with many of these EA structures to demonstrate the same effect in mammalian cells consistently failed (Chapman JD, unpublished data). The discovery that *p*-nitroacetophenone (PNAP) could radiosensitize hypoxic mammalian cells as well as bacterial cells (12) provided the impetus for the majority of studies reviewed in this paper. Radiosensitization by PNAP affected hypoxic cells selectively, occurred at concentrations nontoxic to cells, and was independent of the cell cycle (13). Figure 1 shows typical radiation inactivation data for cells irradiated in various chemical environments. Oxygen is seen to increase the sensitivity of cells by a factor of

~3, at least over the survival levels between 10% and 0.5%. The effect of PNAP in various concentrations on the radiation inactivation rate is shown in figure 1 and its insert. The radiation inactivation rate (k) is determined from the multi-target equation

$$S/S_0 = 1 - (1-e^{-kD})^n$$

where n is assumed constant at ~5.2. Sensitization increases over a specific concentration range (10–500 μM) and reaches a maximum effectiveness with an enhancement ratio

$$(ER = k_{N_2} + drug / k_{N_2})$$

of ~1.5 at concentrations greater than 500 μM. At the time of these experiments it was thought that the EA site within PNAP leading to radiosensitization was the conjugated carbonyl group (11,14). With this in mind, a study was undertaken with various acetophenone derivatives to determine whether effectiveness in radiosensitizing mammalian cells varied systematically with the EA of the carbonyl structure. This study has been reported by Chapman et al (15). It indicates that, whereas the cellular toxicity (measured by cytostatic effect) of the structures studied can be ordered according to the EA of the conjugated carbonyl group, only those structures containing an NO_2 substituent on the benzene ring show radiosensitizing effectiveness at the concentrations tested (fig 2). These data emphasize that the nitrobenzene moiety is the electroactive group in PNAP (16) and that a threshold EA at least equal to nitrobenzene is required to alter significantly the redox environment around the targets of radiation within the mammalian cell. Analogs of nitrobenzene, whose EAs were varied by ring substituents, were tested for effectiveness at the same concentration (500 μM) in radiosensitizing hypoxic mammalian cells (17). For most of the derivatives tested, EA is a useful parameter for ordering their radiosensitizing activity (fig 3). This study led to the selection of some extremely effective hypoxic radiosensitizers. In general, cellular toxicity increases with EA but not in every case. The exceptions to this last observation show that these two effects on the cells do not necessarily result from the same structural group. They offer hope that radiosensitizing activity may be increased independently of cellular toxicity.

Studies performed in parallel with the nitrobenzene investigations showed that some nitrofuran derivatives (currently used clinically as antibacterial agents) are effective radiosensitizers of hypoxic cells (18). This first discovery of radiosensitizing activity in an approved pharmaceutical agent generated hope for an early clinical trial.

The EA of nitrofuran derivatives is greater than that of their corresponding nitrobenzene derivatives (19). This prompted us to test additional derivatives for cellular radiosensitizing activity. Studies with nitrofurazone, nitrofurantoin, and nifuroxime show that these structures act qualitatively like PNAP and oxygen but with ERs intermediate between PNAP and oxygen (20). Screening of numerous nitrofuran structures (generously provided by Norwich Pharmacal, Norwich, NY) resulted in the identification of several structures with radiosensitizing activity near to that of oxygen (21). The most effective cellular radiosensitizers tested to date are nifuraldezone and its water-solubilized derivative (NF–167). At a 250-μM concentration in hypoxia these agents completely mimic the radiosensitizing effect of oxygen in air-saturated solutions (fig 4*a*). This study also indicated that some nitroimidazole and nitrothiazole structures (in clinical use) are effective radiosensitizers of hypoxic cells. The radiosensitizing activities in cells in vitro of the compounds with properties most favorable for animal studies are shown in figure 4*b*.

PHARMACOLOGIC STUDIES WITH CELLULAR RADIOSENSITIZERS

The question now becomes, "Can a radiosensitizing concentration of any of these excellent radiosensitizing structures be established in the target hypoxic cells of solid tumors for the duration of a radiation treatment?" To test this, some pharmacologic experiments were performed with BALB/c mice. These mice had C3H mammary carcinomas growing in their flanks. It was assumed that the con-

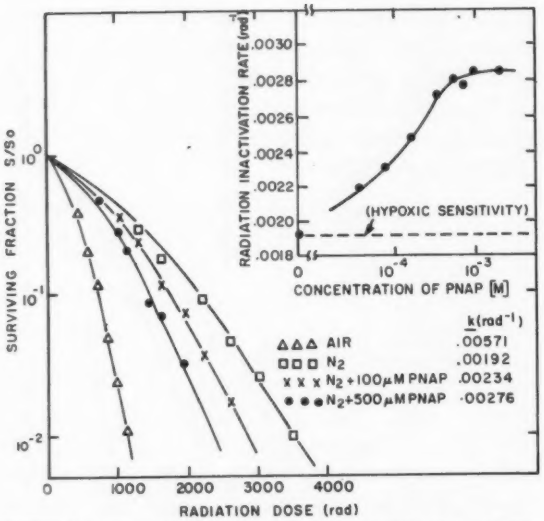

FIGURE 1.—The effectiveness of 100 μM PNAP and 500 μM PNAP in hypoxia and oxygen at air saturation in radiosensitizing the clonogenic capacity of Chinese hamster cells. *Insert*: The concentration dependency of PNAP's sensitizing effect.

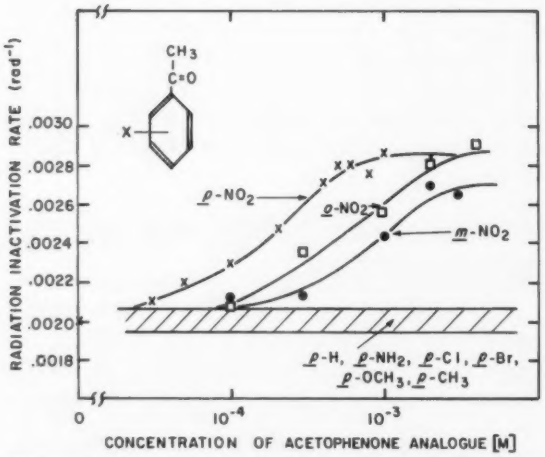

FIGURE 2.—The effect of various concentrations of several substituted acetophenones on the radiation inactivation rate of hamster cells made acutely hypoxic.

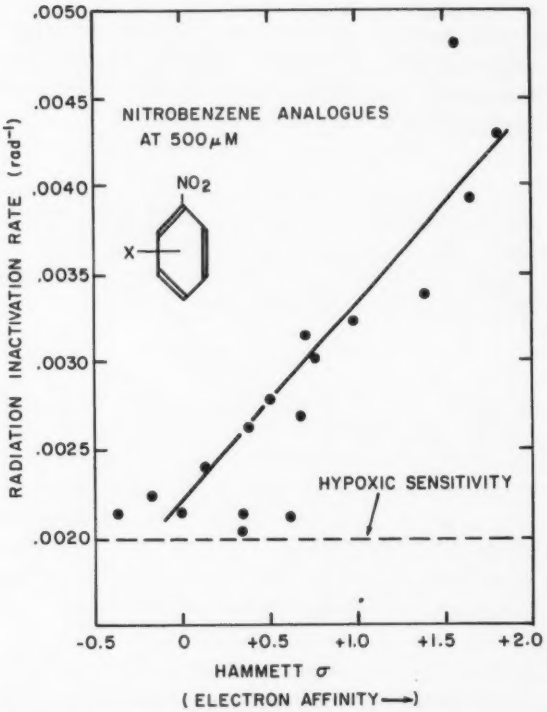

FIGURE 3.—The radiation inactivation rate of hamster cells irradiated in hypoxia with 500 μM of several substituted nitrobenzenes.

centration of drug required to effect radiosensitization of the hypoxic cells within a tumor would be at least that required to radiosensitize cells made acutely hypoxic in vitro. Previous experiments (fig 4*b*) had indicated that, for many drugs, concentrations of ~100 μM were required to obtain ERs of at least 1.5. Most of the pharmacologic publications on the nitroheterocyclic drugs that we are interested in concern much lower drug concentrations (22,23). We had to determine the maximum single dose of the various drugs that could be tolerated by intravenous (iv) and intraperitoneal (ip) administration and then perform drug distribution studies near this acutely toxic limit. For most of the nitrofuran derivatives we tested, this limit is 100–150 and 220–350 mg/kg for iv and ip administration, respectively. Metronidazole and NF-45 are somewhat less toxic than the other derivatives studied.

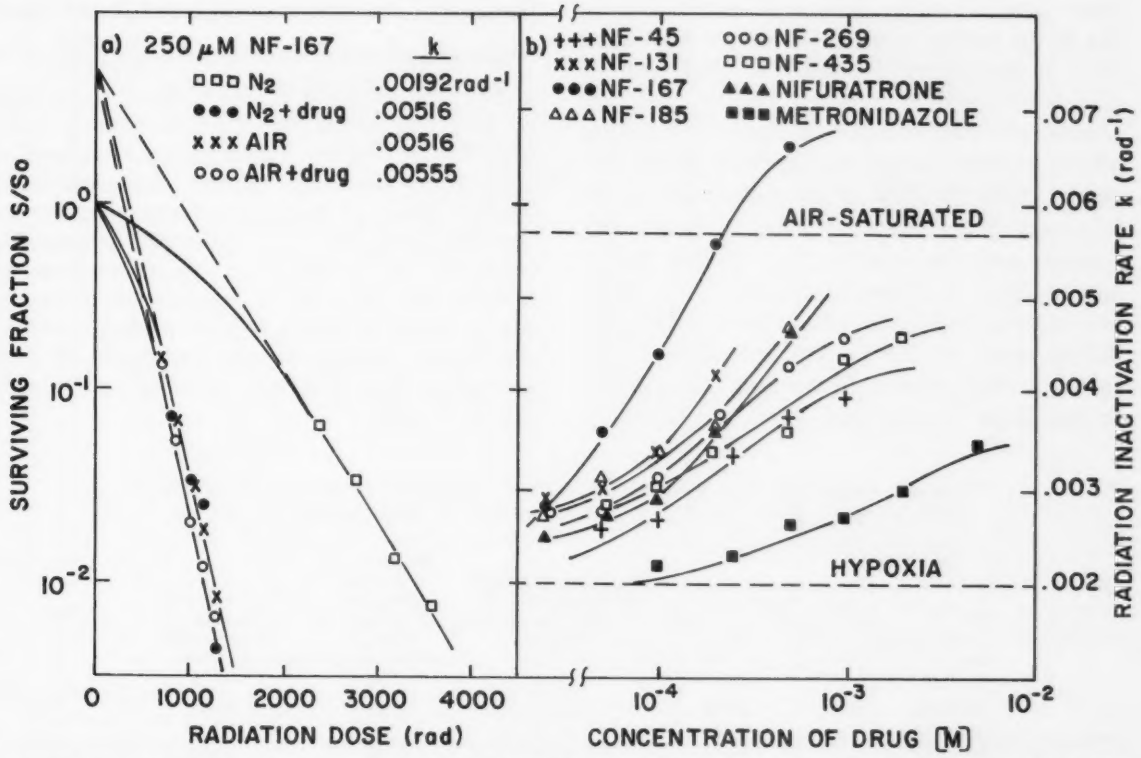

FIGURE 4.—*a*. The radiosensitizing effect of 250 μM NF-167 on both hypoxic and air-saturated hamster cells. *b*. The radiation-inactivation rate of hypoxic hamster cells irradiated in the presence of various concentrations of several nitroheterocyclic drugs.

The drugs were dissolved in sterile physiologic saline at a concentration of 10 mg/ml. Doses of 2.5 mg in 0.25 ml were injected into the tail veins of several mice. At various times after injection the mice were bled from the right common carotid artery and external jugular vein. The blood was collected for estimation of the drug content. In most studies a spectrophotometric assay was used to determine drug concentration. One part of whole blood was rapidly mixed with 9 parts of ethanol and vigorously mixed. After 10 minutes the precipitated blood solids were pelleted by centrifugation and the absorbance of the supernatant was measured at the absorption maximum associated with the ring-NO_2 group of a specific structure. This procedure proved to be a rapid assay for drug concentrations of 30 μM and greater in whole blood. In some studies the loss of drug activity, as measured spectrophotometrically, was compared with the loss of radioactivity associated with ^{14}C-labeled derivatives.

An initial drug concentration in the blood compartment was computed from the weight of the drug injected assuming that the blood of a mouse is ~8% of its weight. Results of drug activity studies with three potential radiosensitizing structures administered iv are shown in figure 5. A rapid loss of drug activity occurs within the first 5 minutes after administration. Therefore, this rapid loss is attributed to dilution of the drug over the total volume (particularly water volume) of the animal. The magnitude of the loss is consistent with a 1/10–1/20 dilution. The radioactivity of ^{14}C-

labeled drugs can be recovered at concentrations equal to that of the blood from at least five different tissues during these short times after administration. After this initial dilution the drugs exhibit exponential decay, each drug with a characteristic half-life. This loss of NO_2-group activity probably results from numerous processes including diffusion into tissues which rapidly metabolize the drug, enzymatic reduction in the blood compartment, and chemical binding (covalent and ionic) to blood components and vessel walls. The relative importance of these processes must relate to the chemical structure of each compound and probably differs even for the limited structures tested in these experiments. In general, the changes in the blood level of each drug can be represented by three parameters: (*a*) the initial concentration of drug in the blood compartment (a computed value); (*b*) the fraction of that initial concentration observed in the blood 5 minutes after administration $\frac{^{c}\text{diluted}}{^{c}\text{INITIAL}}$; and (*c*) the exponential rate of decay (k) shown by its active group over the first hour or half-life ($T\frac{1}{2} = 1n2/k$). These values are given in table 1 for the eight cellular radiosensitizers studied. Many of the most effective sensitizers are too chemically or biochemically reactive to remain in the blood at significant concentrations for 60 minutes. In contrast, metronidazole, which is known to have moderate radiosensitizing activity at concentrations of 500 μM and greater, displays a relatively long half-life in the blood.

TABLE 1.—Parameters that characterize the kinetics of loss of potential radiosensitizers from the blood compartment of BALB/c mice after iv administration of 2.5 mg

Compound	Initial concentration in blood (μM)	Fraction of initial concentration in blood after 5 min	Half-life of active group in blood (min)	Octanol/water partition coefficients
NF-45	8600	0.10	21	~15
NF-131	3220	0.05	30	0.60
NF-167	2960	0.04	11	0.035
NF-185	1600	0.05	24	0.047
NF-269	3080	0.11	25	0.35
NF-435	3220	0.102	19	0.57
Nifuratrone	5600	0.065	15	0.63
Metronidazole	6500	0.127	~80	0.84

Experiments were performed with some of the same sensitizers administered ip. The resultant blood levels are shown in figure 6. Both metronidazole and NF–45 can be maintained in the blood at high concentrations for at least 60 minutes when administered in this manner.

We then attempted direct measurements of the drug in solid tumors. For these we used a similar ethanol extraction procedure on minced tissues from the periphery and from the interior of the tumors grown to 1–2 cm in diameter in mice. The results from studies with four compounds are shown in figure 7 (*a–d*). Both metronidazole and NF–45 diffuse into the tumor tissue and equilibrate at the concentration observed in the blood within 60 minutes. The two compounds, 2-hydroxymethyl-5-nitrofuran and NF–269, can never be recovered from the tumor tissue in a concentration equal to that of the blood. Furthermore, the rapid decay of these latter drugs in the blood and in the tumor tissue makes them unlikely candidates for clinical sensitizers. Preliminary data with NF–131 indicates that equilibration between blood and tumor tissue may occur but only after more than 60 minutes. This assay has proven useful in determining which compounds penetrate solid tumor tissue and equalize there with their measured blood concentrations. Both metronidazole and NF–45 have now been shown to radiosensitize the hypoxic cells of tumors

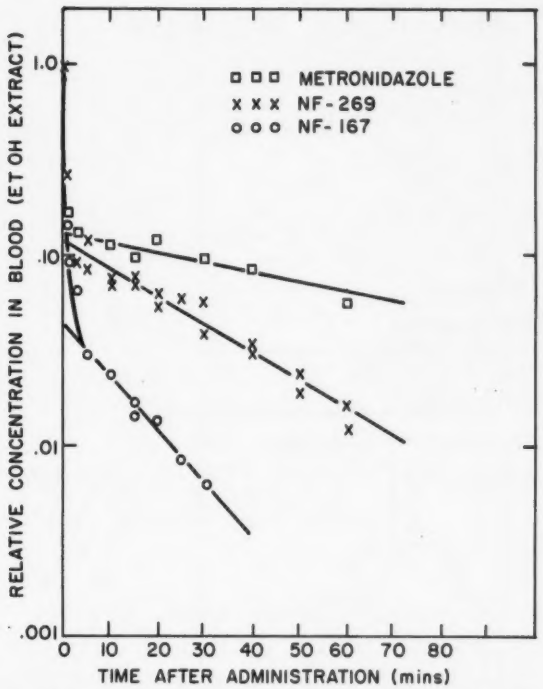

FIGURE 5.—The relative concentration of three cellular radiosensitizers in the blood of BALB/c mice after administration of 2.5 mg iv. The initial concentration in the blood compartment was computed assuming the blood to be ~8% of the mouse weight.

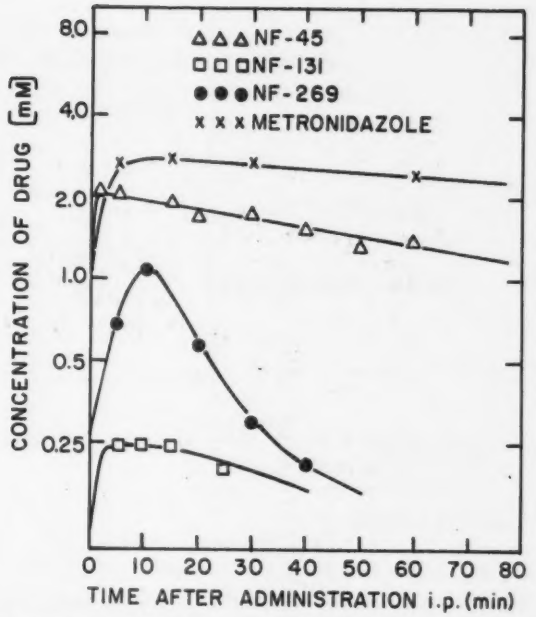

FIGURE 6.—The absolute concentration of four cellular radiosensitizers in the blood of mice after ip administration of 10 mg NF-45, NF-269, and metronidazole and 5 mg NF-131.

treated in vivo, whereas neither NF–167 nor NF–269 is effective (24).

Preliminary pharmacologic studies with high doses of metronidazole have been performed with human cancer patients in preparation for a controlled clinical trial to determine its effectiveness in radiotherapy (25).

MECHANISM OF CHEMICAL RADIOSENSITIZATION

The radiation inactivation of the proliferative capacity of mammalian cells is an extremely complex process. It is schematically represented in figure 8. This gives an oversimplified view, but we hope it conveys the time scale for the events involved in the expression of radiation damage and an idea of the ways in which the chemicals might extend their influence. From the outset of our studies, we have searched for compounds which selectively radiosensitize hypoxic cells. Such compounds may mimic molecular oxygen in the radiation-induced chemical reactions of molecules that are vital for cell reproductive integrity. Our present understanding of radiosensitization by nitroheterocyclic drugs indicates that they act like oxygen.

Energetic electromagnetic radiations, such as X- and γ-rays, interact with the molecules of a living cell and eject therefrom energetic electrons. These electrons distribute their energy through ionization and excitation of additional molecules along a track not far removed from the primary site of interaction. Ionizations and excitations occurring in vital cellular molecules with resultant alteration to their chemical structures have been classified as direct effects. However, much of the energy absorbed by a cell is absorbed in water molecules. This results in highly reactive free radi-

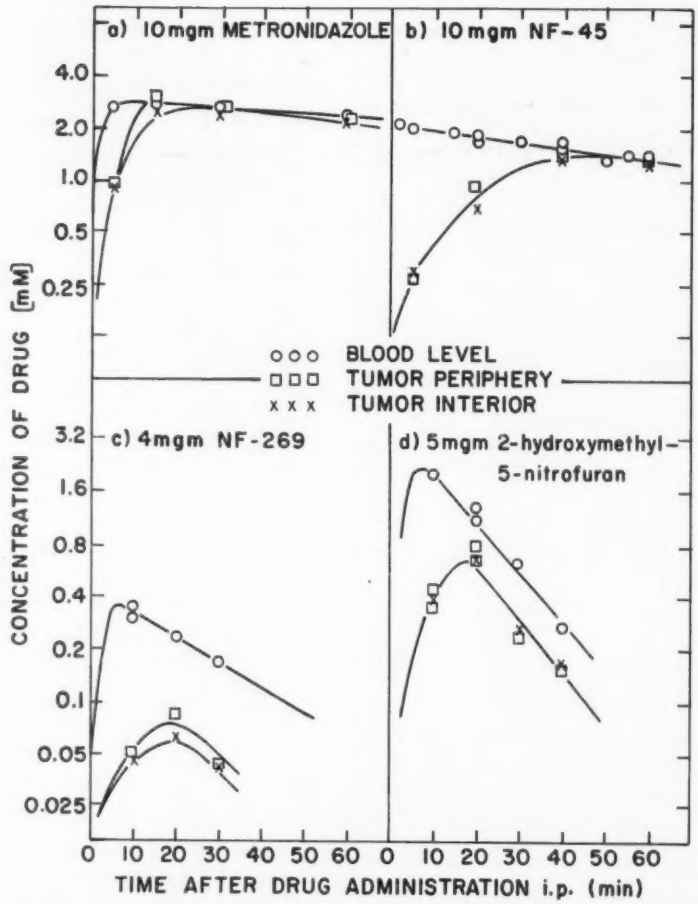

FIGURE 7.—The absolute concentrations of sensitizers recoverable from the blood, tumor periphery, and tumor interior after ip administration of: *a*) 10 mg metronidazole; *b*) 10 mg NF-45; *c*) 4 g NF-269; and *d*) 5 mg 2-hydroxymethyl-5-nitrofuran.

cal species. These free radicals diffuse to and react with vital cellular molecules leading again to altered chemical structures. Such events have been classified as indirect effects. The most important free radical species generated in the radiolysis of water are H, OH, and e^-_{aq}.

Studies using compounds which compete with cellular target molecules for OH (26, 27) show that ~60% of the rate at which air-saturated hamster cells are inactivated by radiation depends on the indirect action of OH. With cells made acutely hypoxic, ~30% of their inactivation rate depends on the indirect action of OH. By the use of various alcohols and DMSO as OH scavengers, the reaction rate between OH and the cellular target or targets is estimated to be $\sim 9 \times 10^8$ seconds^{-1}. Consequently, relatively high concentrations of these compounds are required to demonstrate cellular radioprotection. Many OH scavengers are too toxic to cells to be useful as radioprotectors. We are not yet able to demonstrate, using e^-_{aq} scavengers, any component of cell inactivation associated with e^-_{aq}. The reaction rate of the cellular target(s) with e^-_{aq} is expected to be as great as that with OH. Our inability to demonstrate cellular

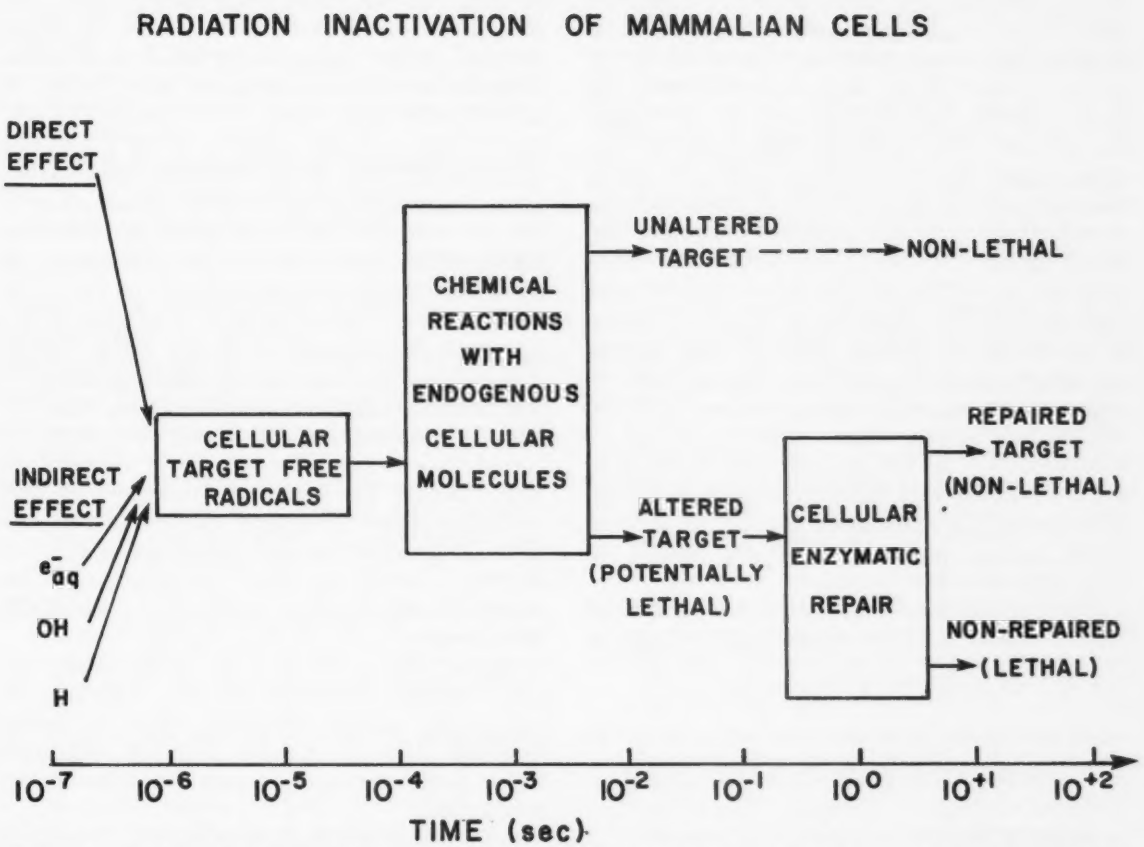

FIGURE 8.—A schematic representation of ways in which ionizing radiation can induce altered cellular targets that subsequently lead to loss of proliferative capacity.

radioprotection with e^-_{aq} scavengers may indicate that these compounds become toxic to cells at concentrations well below those required to compete effectively with the target(s) for e^-_{aq}. In light of these studies and of additional reports (28,29), we tentatively apportion the radiation inactivation of air-saturated cells to direct effect, to OH, and to e^-_{aq} and H as 30%–40%, ~60%, and $\leq$ 10%, respectively.

Oxygen (30) and nitrofurazone (31) are known to react very quickly with both OH and e^-_{aq}. Yet, the concentrations of each required for radiosensitization in a mammalian cell are not adequate to compete effectively with the cellular target(s) for either radical species. Radiosensitization by nitroheterocyclic drugs is thus thought to result from reactions between the sensitizer and cellular target free radicals. The majority of these would be neutral radicals generated by direct action and OH. Studies with nitrofurazone show that it binds to various biomolecules and whole cells when irradiation takes place in the absence of oxygen (20). This binding results predominantly from the reaction between nitrofurazone and a neutral radical generated in the target molecules by the indirect action of OH (31). Both sensitizer binding to and electron transfer from neutral target radicals may result in an altered chemical structure. Both may play a role in cellular radiosensiti-

zation (32). When oxygen reacts with neutral radicals generated in various target molecules, oxygen binding (or peroxidation) is favored (33). The EA radiosensitizers added to a hypoxic cell make oxidation of neutral target radicals more likely and they compete for these radicals with endogenous radical-reducing species (26). This reaction, basic to the oxygen-fixation hypothesis (34–36), is schematically represented in figure 9. Neutral radicals generated in the cellular target(s) by direct action and by OH are important intermediates in cellular inactivation. They account for ~82% of the inactivation observed under air-saturated conditions (26). Radical anions are generated in the cellular target(s) by reactions with e^-_{aq} with equal efficiency. These will be oxidized by rapid electron transfer to substances whose EA exceeds that of the target material. In this manner, oxygen and other endogenous EA substances can act to restore target radical anions (potentially lethal) to their original state.

The radiobiologic target within a cell most readily linked with its reproductive death is the DNA. Numerous lesions in the DNA of irradiated cells have been characterized (*see* ref 37). Among them are strand breaks; the production of these is enhanced in the presence of oxygen by a factor of 3–4 (38–40). Many strand breaks produced by radiation in air-saturated cells result from the indirect action of OH with the cellular DNA (41). The nitroheterocyclic radiosensitizers mimic oxygen in requiring the indirect action of OH for the majority of their effect on cell viability (26). They enhance the production of detectable strand breaks produced in the DNA of mammalian cells irradiated in hypoxia (39). Not only is the quantity of the DNA breaks changed in the presence of these sensitizers, but recent experiments with model chemical systems indicate that the quality (or chemical specificity) of the DNA breaks is changed as well (42,43). The ultimate expression of any radiation-induced chemical change in the cellular DNA depends upon the action of cellular enzymic repair systems. Therefore, quality changes in the chemical specificity of the DNA lesions are of extreme importance.

The nitroheterocyclic drugs bring about cellular radiosensitization in the absence of oxygen by radical oxidation. This increases chemical damage in vital cellular molecules whose integrity is important for cellular reproduction.

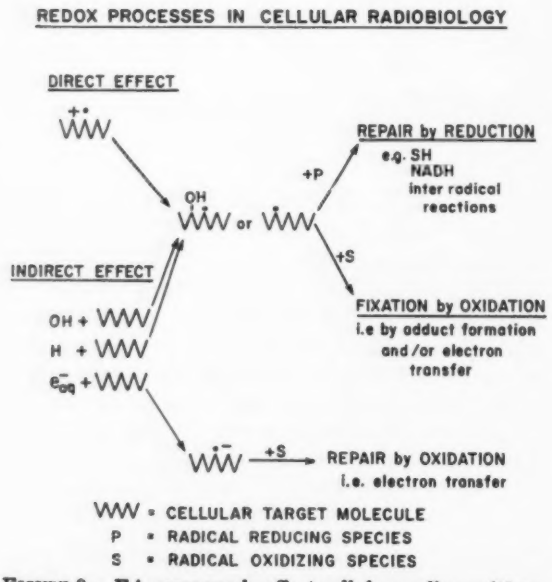

FIGURE 9.—EA compounds effect cellular radiosensitization predominantly by the oxidation of neutral target radicals.

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Interferon: Its Inducers and Some Aspects of Host Resistance in Malignancy

Thomas C. Merigan [1]

SUMMARY

In laboratory animals, both endogenously induced and passively administered interferon have antiviral effects against a variety of infections including those caused by oncogenic viruses. Such beneficial effects are often most striking when the interferon is administered prophylactically. Occasionally, interferon is also active therapeutically and its continued administration is required for maintenance of the beneficial effect. A number of possible explanations exist for the mechanism of these effects. These include prevention of recruitment of new cells into the transformed state, a direct oncolytic effect, or a selective inhibition of tumor cell division. In addition, a cooperative effect of interferon with tumor immunity is possible in that interferon can potentiate the cytotoxicity of sensitized lymphocytes and enhance phagocytic activity of unsensitized macrophages. The reported beneficial effects of interferon inducers in animal models are further complicated by additional noninterferon-mediated effects of the inducers on host defenses. Recently, an inhibitory action of interferon has been noted on animal tumors not associated with virus, as well as on certain other rapidly dividing normal host cells. Because of these findings and its safety and efficacy when employed in patients with infectious diseases, clinical testing of human leukocyte interferon in patients with tumors has been initiated. Present data are restricted to studies of interferon pharmacokinetics and safety, as well as its effect on specific host defenses, in comparison to other metabolic antagonists. In short-term studies high levels of circulating interferon can be obtained by intravenous or intramuscular administration of partially purified human leukocyte interferon without any limiting effects. Interferon is produced by sensitized lymphocytes when they are stimulated on an immune-specific basis, as well as a wide variety of cell types when they are infected with viruses or other intracellular parasites. Hence, these interferon trials may be interpreted as a form of immunotherapy. Bacillus Calmette-Guérin, specific lymphocyte transfer factor, irradiated tumor cells, and immune or nonspecifically activated lymphocytes and interferon inducers including endotoxin are all other potentially related useful approaches for the control of cancer in man.

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[1] Division of Infectious Diseases, Stanford University School of Medicine, Calif.

EFFECT OF INTERFERON ON TUMORS

Interferon is an antiviral substance produced as a natural response of vertebrate cells to viral infection and other stimuli (fig 1). As it is nontoxic, nonantigenic (in the homologous species), and very potent on a weight basis, it has appeal for use in human medicine. It recently has been observed to have antitumor effects in vivo and in vitro, and hence may also be a clinically useful antineoplastic agent.

Animal Experiments

In laboratory animals interferon has been noted to have antiviral effects on a wide variety of infections including those caused by cytocidal and oncogenic viruses (1). Most of the work with the latter has involved murine tumor viruses including studies of the Friend-AKR-Rauscher complex (2–7), mammary tumor virus (8), Moloney sarcoma virus (9,10), and radiation lymphoma virus (11) (table 1). Studies with concentrated but partially purified materials have demonstrated both prophylactic and less striking therapeutic effects. It is reasonable to assume that the interferon in the preparations administered is responsible for the beneficial effects. In general, the beneficial effects were only maintained with the continued administration of interferon. No toxic effects or growth inhibition were observed in mice maintained on similar doses of interferon for their lifetime (12). A lesser number of observations exist in other species, but Rous sarcoma virus tumors in chickens (13), polyoma tumor formation in hamsters (14), and Shope fibroma production in rabbits (15) have all been reported to be inhibited by prior application of the appropriate homologous interferon preparations.

TABLE 1.—Viral-induced tumors inhibited by interferon preparations

Animal species	Virus	
	Class	Name
Mouse	Leukovirus	Friend-AKR-Rauscher virus
	Leukovirus	Mammary tumor virus
	Leukovirus	Moloney sarcoma virus
	Leukovirus	Radiation lymphoma virus
Chicken	Leukovirus	Rous sarcoma virus
Hamster	Papovavirus	Polyoma virus
Rabbit	Poxvirus	Shope fibroma virus

The mechanism by which these interferon preparations affect tumors is not clear at present. A number of in vitro studies (1) indicate that interferon, if given prophylactically, can inhibit tumor virus infection (table 2). Hence, it was originally postulated that interferon achieves an in vivo antitumor effect on established infections by preventing the recruitment of new cells into the transformed state. However, several alternative explanations are suggested by other observations (fig 2). In AKR leukemia of mice, Kassel and his associates (6) have observed a direct oncolytic effect of interferon occurring within ½ hour after it is injected. Paucker, Cantell, and Henle (16) first observed an in vitro inhibitory growth effect of interferon on L cells which has been extended by Gresser and his group and other investigators to a variety of malignant cells (17) as well as certain rapidly dividing normal embryo cells (18–20). A cooperative effect of interferon with immunity has been observed in that it potentiates the cytotoxic effect of sensitized lymphocytes (21–23) and enhances the phagocytic activity of unsensitized macrophages (24). Another recently identified possibility is that interferon might accelerate the rejection of tumors by increasing their immunogenicity, since murine interferon preparations have been noted to enhance the expression of surface antigens in L1210 cells (25).

a. A glycoprotein of 20,000–30,000 molecular weight (mol wt) with additional higher mol wt species of 40,000–60,000, 80,000–90,000, etc; specific activity $\geq 10^9$ units/mg protein; nonantigenic in the homologous species.

b. Synthesized in vivo and in vitro by all vertebrates studied in response to viral infection as well as to other intracellular parasites, polyanions (polycarboxylates, polynucleotides, etc), endotoxin, and certain low mol wt bis basic organic compounds. Also produced as a part of cell-mediated immune (mitogenic) response by lymphocytes.

c. Acts intracellularly in a characteristic species-specific fashion against both RNA and DNA viruses (inhibiting viral messenger RNA function and/or production) as well as inhibiting more complicated intracellular parasites (protozoa, rickettsia, etc). Antiviral action depends upon the cellular production of RNA and protein.

d. Nontoxic in that it does not affect normal cell functions (energy metabolism or macromolecular synthesis), but it inhibits rapidly dividing cells, including tumor cells.

FIGURE 1.—Summary of the properties of interferon.

a. Antiviral effect prevents further transformation

b. Direct cytocidal action (oncolysis)

c. Growth-inhibitory activity on rapidly dividing cell

d. Potentiation of cytotoxic effect of sensitized lymphocytes

e. Enhancement of phagocytic activity of macrophages

f. Enhanced expression of tumor-specific surface antigens

g. Inhibition of blocking antibody production

FIGURE 2.—Possible mechanisms by which interferon inhibits tumors.

Interferon inducers have also been noted to have beneficial effects in virus- or carcinogen-induced malignancy in vivo (26). Here, a wide variety of immunologic or nonimmunologic related mechanisms could possibly underlie the positive effects, including many that do not directly involve interferon (27). In fact, in only one instance has interferon been shown clearly to underlie the antitumor effect of the inducer (10). The timing of inducer administration is most critical, as is the age of the host (28), in achieving useful effects. Enhancement of tumor growth has also been reported following inducer administration (29). The interferon inducer approach is more difficult to manage for several reasons: (*a*) the two-step nature of inducers is more inherently complicated (interferon production followed by its action in the host) and hence prone to failure compared to the direct action of passively administered interferon; (*b*) inducers have additional significant toxicities not associated with interferon; and finally (*c*) hyporeactivity to interferon stimulation occurs consistently with repeated injections of inducers. Therefore, perhaps it is not surprising that results (such as synthetic double-stranded RNA) in patients with viral or neoplastic disease have not been striking to date (30–33).

TABLE 2.—Oncogenic viruses inhibited in vitro by interferon preparations

RNA virus	DNA virus
Rous sarcoma virus	Polyoma virus
Feline leukemia virus	Shope fibroma virus
Friend leukemia virus	SV40 virus
Rauscher leukemia virus	Adenovirus
Moloney sarcoma virus	Herpesvirus saimiri

Interferon preparations have also been reported to inhibit the occurrence of spontaneous tumors in mice (7,9), methylcholanthrene-induced tumors in mice (34) and monkeys (35), and various transplantable tumors in mice (36), including a metastasizing tumor (Lewis lung tumor) (37). In several of these models therapeutic as well as prophylactic effects have been observed. However, parallel with such observation of interferon effects on tumors not necessarily associated with viruses, several reports have appeared on the in vitro effects of interferon on lymphocyte DNA synthesis (38) and multiplication of rapidly dividing normal cells including bone marrow cells (39), human diploid cells (19), and mouse kidney cells (18). Most recently, large doses of interferon have been noted to partially diminish T-cell-mediated immunity in mice (40) as well as the number of antibody-forming spleen cells in mice undergoing primary immunization (41). These latter observations of immunosuppression are not surprising considering the similar problems seen with the antineoplastic antimetabolites. However, they make it mandatory to perform careful observation of immunity parameters in patients undergoing interferon therapy.

The lack of effect of interferon on the biosynthetic functions of nondividing cells (13, 42–45) is an important favorable point to consider in assessing its potential clinical antitumor application. When studied in vitro in much higher doses than required for antiviral effects interferon has been observed to inhibit protein synthesis (46) and to impair the translation of the endogenous messenger RNA (47) in a cell-free system. However, aryl hydrocarbon hydroxylase in mouse cells is activated by purified interferon (48), but steroid induction of tyrosine transaminase is not influenced by doses of interferon which are active in inhibiting virus growth in the same cells.[2] All of these in vitro findings support the contention that interferon should have a wide enough therapeutic ratio for at least short-term clinical trials and fit with its lack of toxicity in animals (5,7,12,49).

[2] Colby C. The establishment of the interferon-inducing antiviral state. Presented at Table Rounde Roussel UCLAF, Paris, 1973.

A recent paper (50) has described a synergistic action of an antiviral drug, BCNU,[3] and interferon against a murine leukemia. Although the mechanism of the effect was not clear, interferon given at any time during the usual 2–3-week remission period produced by the drug would protect the animals from relapse for over 80 days. Given the diverse areas listed above when interferon's antitumor action may take place and its relative lack of toxicity, it may be possible to use it in combination with a cytostatic agent for beneficial clinical effects.

Clinical Studies

The earliest efforts at administration of interferon to patients with malignancy were those of Falcoff and associates (51) who gave modest doses of amnion-produced human interferon to 18 patients with acute leukemia. Although they were favorably impressed with the results (including lack of toxicity), they were careful to point out that their study was not controlled and allows no firm conclusions. Several viral diseases of the eye and respiratory tract have also been reported to respond favorably to human leukocyte interferon in controlled trials in the Union of Soviet Socialist Republics (1). Unfortunately, few details of these studies are presently available.

More recently, data have been accumulated on the results of regular intramuscular (im) injection of human leukocyte interferon into patients with a wide variety of late stage tumors. Strander et al (52) have treated 28 patients with up to 3×10^6 units (three times weekly) for 2½ years with no limiting side effects. Fever was noted in most patients for the first few injections, and late in the course of treatment antibodies were noted to some of the contaminating inactive virus used to stimulate the leukocytes and the proteins of the chick cells in which it was grown, but not to the interferon itself. The wide variety of patients studied and the uncontrolled nature of this trial preclude any conclusions regarding efficacy, but indicate that this dosage is virtually free of toxicity. The patients still under treatment by Strander et al at the Karolinska Hospital include several with Hodgkin's disease (five patients), acute leukemia (three patients), osteogenic sarcoma (nine patients), and others with various solid tumors. We topically employed 14×10^6 units of the same interferon preparation used by Strander et al in the nose to prevent common colds in volunteers. In a randomized, double-blind placebo controlled trial interferon was successful in protecting normal individuals from rhinoviral infections without any side effects (53). Currently this material is also being given parenterally to patients with malignancy and acute disseminated varicella-zoster (V-Z) infections in doses of $10–50 \times 10^6$ units every 12 hours.[4] So far, 11 patients ranging in age from 7 to 54 years have been treated. Pharmacokinetic studies comparing intravenous (iv) and im dosing reveal im administration to be as convenient and as efficient as iv infusion in producing sustained blood levels. The present dosage scheme will produce circulating interferon levels of up to 1000 units for 3 days with no side effects except fever. It is of particular importance that no suppressive effects were observed on host defense responses to the infecting virus (V-Z complement-fixing antibodies, vesicle fluid cellular and interferon responses).

Interferon Preparation

All of these recent studies are made possible through the human interferon production efforts of Cantell and his co-workers at the State Serum Institute in Helsinki. Their methods for leukocyte interferon production have improved and expanded each year (54). In 1972 they produced 42 pools with a mean of 50,000 units/ml for a total of 10^{10} units. These workers have also designed a simple but highly selective purification procedure involving ethanol precipitation and acid solubilization which gives a hundredfold purification of interferon with respect to other proteins with a 50% yield (54). Because of the greater expense of and lack of clinical experiences with either human diploid or lymphoblastoid cell interferon, these are

[3] BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

[4] Merigan TC, Fried RP, and Jordan GW. Personal communication, 1973.

less desirable alternatives for trials than human leukocyte interferon.

EFFECT OF INTERFERON INDUCERS AND RELATED COMPOUNDS ON TUMORS

Animal Experiments

Because of encouraging results in animal studies, interferon inducers are being explored in man for clinical antitumor effect (*see* Clinical Studies below). However, there are complicated side effects and yet-to-be defined conditions for favorable effects such as proper dosage level, timing, and route of administration. For example, Gadzar et al (as was mentioned previously) have reported several studies in which tumor enhancement was obtained with certain dosage schemes of several types of inducers which were otherwise tumor inhibitory (29). There are also antiviral effects known with the inducers that do not involve the interferon system but involve other aspects of immunoenhancement, etc. The ready availability and inexpensiveness of the synthetic inducers of interferon have stimulated animal tumor protection experiments (1) which are too numerous to review comprehensively here. Examples of such studies include the work of Regelson (55) in preventing Friend virus leukemia in mice with pyran copolymer (a polycarboxylate plastic), that of Levy et al (56) in inhibiting a variety of tumors in mice with double-stranded RNA, and that of Rhoads et al (57) in suppressing several transplantable solid tumors in rats with the administration of tilorone.[5]

Clinical Studies

The early observations by Coley (58) at the turn of the century that bacterial toxins would benefit patients with malignancy might represent the effect of an interferon inducer. The studies of Mathé (59) with bacillus Calmette-Guérin represent a modern-day application of bacterial products to the treatment of neoplasms. Viruses have also been utilized for oncolytic effects in man without sufficiently striking positive results to outweigh the complexities and dangers involved in their use (60,61). Although pyran copolymer was observed to have toxicity in its first trials in man (62), it is still possible that newer less toxic preparations will merit further clinical trials. As mentioned previously, the synthetic double-stranded RNA, poly (rI) · poly (rC) [6] has been studied previously for antineoplastic effect in man by several groups (30–32). Although it may be too early to judge its clinical efficacy, it is not yet clear that its interferon-inducing or immunoenhancing effect will prove clinically useful. Ayre and his associates (33) have reported enthusiastically on an uncontrolled trial of poly (rI) · poly (rC) injected directly into cervical carcinoma in situ, but this will need further study.

As sensitized lymphocytes respond to antigens with interferon production as well as other mediators (63), the current efforts being made with the use of lymphocyte "transfer factor" in man could also be viewed partially as interferon induction (64,65). The same point would hold for a number of methods of immunotherapy discussed previously by others at this symposium. Such work is, however, in too early a stage to evaluate its real clinical significance.

CONCLUSION

It appears that we shall see efforts to utilize the immunoenhancement and the interferon mechanisms in human neoplasia in the years to come. With the broad range of synthetic and natural products with the property of interferon induction, some sort of practical application should be possible. However, it may well be that the nonantiviral, immunoenhancing, or "non-specific" aspects will prove to be the most useful, rather than interferon stimulation per se. Major stumbling blocks in devising a strategy for use of interferon and its inducers in cancer include the lack of in vitro assays to predict in vivo efficacy, as well as the present lack of precise understanding of the complex role of immunity in host resistance to tumors. However, appropriately moni-

[5] Tilorone: NSC-143969; fluoren-9-one, 2,7-bis[2-diethylamino)ethoxy]-,dihydrochloride.

[6] Poly (rI) · poly (rC): NSC-120949; 5′-inosinic acid, polymers, complex with 5′-cytidylic acid polymers (1:1); polyinosinic:polycytidylic acid.

tored and controlled clinical trials can be undertaken to establish indications for therapy, in vitro correlates of in vivo action, and satisfactory endpoints of efficacy.

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Interference With Formation and Expression of RNA Tumor Virus Information: Rationale and Experimental Results

Robert C. Gallo, Robert E. Gallagher, Robert C. Ting, R. Graham Smith, David H. Gillespie, and Alan M. Wu [1]

In this report, we would like to review broadly some of our concepts about RNA tumor viruses: their origin, some features of their biochemistry and mode of transmission, evidence for subviral components in human leukemic cells, and approaches to inhibit their replication and function. More critical and detailed reviews pertaining to the molecular biology of RNA tumor viruses have recently been published or are in press elsewhere (1–4).

ORIGIN OF RNA TUMOR VIRUSES

There is evidence which indicates that the type-C RNA tumor viruses evolve from cellular genes. Information related to these viruses may exist in an incomplete form and subsequently may evolve into frank virus particles (5). The function of this information in normal cells is not known, but for several reasons it is suspected that it is related to the process of embryogenesis and that it originates from cytoplasmic gene (DNA) synthesis, eg, type-C virus and/or virus markers are found in some embryonic cells. The viruses which arise directly from normal cells are called endogenous type-C viruses, and molecular hybridization experiments show that their genetic information can be accounted for by information pre-existing in the normal host cell (2). We have called them class I viruses. Good examples of these are the feline endogenous virus, RD-114, and the apparent primate endogenous type-C virus identified in the placentas of baboons (6–8). [2] Most, if not all, true endogenous viruses (unmodified) are not directly tumorigenic for their own species. We believe that the frankly oncogenic type-C viruses (we call them class II viruses) evolve from the endogenous viruses. There are major differences between the two types. For instance, the reverse transcriptases of feline sarcoma virus (FSV) and feline leukemia virus (FeLV) are immunologically different from RD-114 reverse transcriptase (9), and reverse transcriptases from the endogenous primate placental virus of baboons (7) and from rhesus monkeys [2] are immunologically different from the reverse transcriptases from the primate type-C viruses associated with neoplasms, ie, in primates, simian sarcoma (woolly monkey) virus (SiSV) and gibbon ape lymphoma (GAL) virus. Moreover, molecular hybridization experiments show little, if any, relatedness between the endogenous viruses and these tumorigenic viruses. Some recent hybridization experiments indicate that the RNA tumor viruses change their genetic information most profoundly when they infect a new cell.[3] This might be explained by infidelity of reverse transcriptase (10) or by recombination of the newly made viral DNA with DNA of the newly infected cell. During infection of a new cell, the endogenous type-C viruses may undergo considerable genetic change and in this process accumulate information that subsequently may be tumorigenic. We propose then that "viral" genes are in all cells and that certain RNA "tumor" viruses have evolved relatively recently from these genes, but we do not think that all information for oncogenesis pre-exists

[1] Laboratory of Tumor Cell Biology, Experimental Therapeutics, Division of Cancer Treatment, National Cancer Institute, and Bionetics Research Laboratories, Bethesda, Md.

[2] Mayer RJ, Smith RG, and Gallo RC. Reverse transcriptase in normal rhesus monkey placenta. Submitted to *Science*, 1974.

[3] Gillespie DM, Saxinger WC, East JL, et al. Host effect on RNA tumor virus genetic information. Submitted to the *Proceedings of the National Academy of Sciences USA*, 1974.

in all somatic cells of vertebrates as suggested by the oncogene hypothesis (11).

Type-C viruses have a bouyant density of 1.16 and contain more densely banding inner structures (cores) containing an RNA genome, 50S–70S in size, which can be dissociated into apparent subunits of approximately 28S–35S. Also associated with the core is the viral-specific DNA polymerase, reverse transcriptase. Many other virion-specific proteins have also been identified (1).

MODE OF INFORMATION TRANSFER BY RNA TUMOR VIRUSES

The transmission of information from RNA tumor viruses may occur by infection of animals or cells from without (horizontal spread) or in some specific cases by vertical transmission when the DNA provirus made from the virus RNA is integrated into a gamete (transmission in this case is from parent to progeny). One can also speak of vertical transmission when cells which contain integrated viral information divide, in which case the daughter cells also contain the viral DNA genome. Congenital infection is known to occur in some species, eg, avian leukosis virus in chickens (12). In a sense this is a special case of vertical transmission at the whole animal level, but mechanistically it is horizontal transmission since it requires infection from without rather than transmission of endogenous information. These are all schematically depicted in figure 1. Not all these modes of transmission are known for each virus or each species, but they are all theoretically possible, all do occur in some species (eg, chickens), and each type is known to occur in different mammals.

Several processes are known to occur when these viruses infect and transform a cell and replicate in it. These are schematically illustrated in figures 2–4. The virus absorbs to cell membranes, penetrates, uncoats, and synthe-

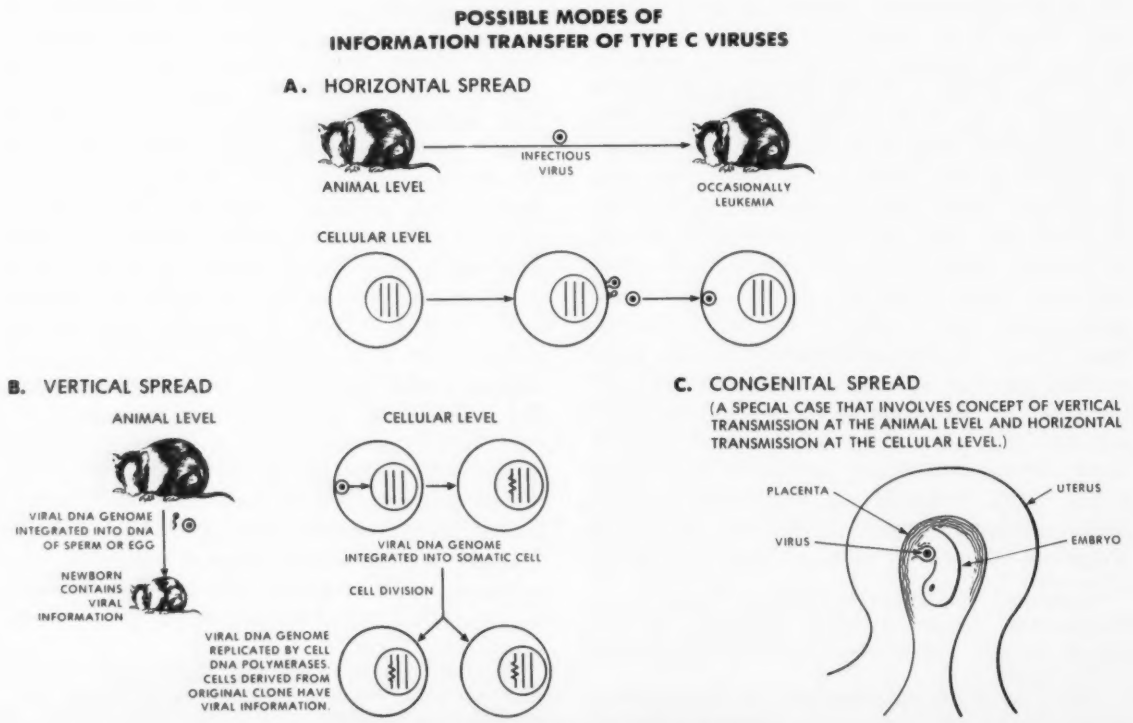
POSSIBLE MODES OF
INFORMATION TRANSFER OF TYPE C VIRUSES
A. HORIZONTAL SPREAD
INFECTIOUS VIRUS
ANIMAL LEVEL
OCCASIONALLY LEUKEMIA
CELLULAR LEVEL
B. VERTICAL SPREAD
ANIMAL LEVEL
CELLULAR LEVEL
VIRAL DNA GENOME INTEGRATED INTO DNA OF SPERM OR EGG
NEWBORN CONTAINS VIRAL INFORMATION
VIRAL DNA GENOME INTEGRATED INTO SOMATIC CELL
CELL DIVISION
VIRAL DNA GENOME REPLICATED BY CELL DNA POLYMERASES. CELLS DERIVED FROM ORIGINAL CLONE HAVE VIRAL INFORMATION.
C. CONGENITAL SPREAD
(A SPECIAL CASE THAT INVOLVES CONCEPT OF VERTICAL TRANSMISSION AT THE ANIMAL LEVEL AND HORIZONTAL TRANSMISSION AT THE CELLULAR LEVEL.)
PLACENTA
UTERUS
VIRUS
EMBRYO

FIGURE 1.—Transmission of type-C viruses.

sizes a DNA transcript (the provirus). This is catalyzed by reverse transcriptase. If this DNA provirus is integrated into the host cell (fig 2) it may be propagated to progeny cells. Some of the enzymes required in this process are depicted in figure 3. Reverse transcriptase is essential for the first step (synthesis of the provirus). We know all infectious RNA tumor viruses contain this DNA polymerase. Other evidence that the enzyme is essential has come from studies with polymerase mutants and inhibitors. Viruses with inactive reverse transcriptase from mutation (13) or by deliberate inhibition (14) are not infectious in vitro or in vivo (15). It is now known from studies with temperature-sensitive mutants that the genetic information for reverse transcriptase is carried by the virus RNA genome (Baltimore D, personal communication). That the genetic information from the RNA genome of these viruses is converted into DNA was clearly demonstrated (16) and subsequently confirmed (17) (Temin H, personal communication). These studies showed that *DNA* isolated from nuclei of infected cells could in turn infect normal cells.

To propagate the viral DNA into progeny cells, cell DNA polymerases are presumably utilized (fig 3). For the viral DNA genome integrated into the host cell (figs 2–4) to direct synthesis of viral RNA (and hence viral proteins), cellular RNA polymerases are probably used. After formation of the viral RNA, poly(A) sequences are added. This process can be interrupted by cordycepin (3′-deoxyadenosine, NSC-63984) (18) (fig 4). The integrated viral DNA may stay dormant (not expressed) in which case there may be no phenotypic change in the cell and no virus production. Partial expression may occur with viral RNA and viral proteins detected and with or without phenotypic change (eg, transformation) of the cell. Complete expression may occur with virus production with or without transformation.

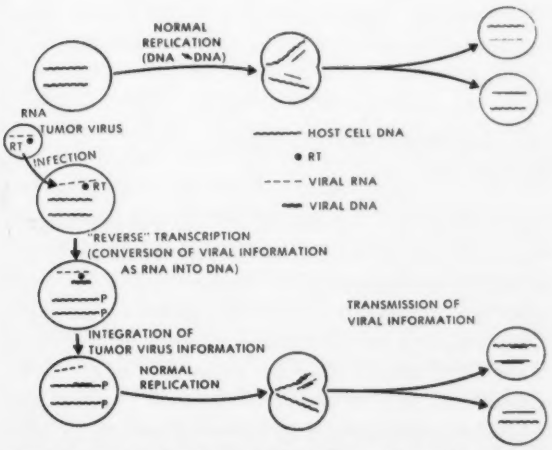

FIGURE 2.—Replication of DNA in uninfected cells and after infection by type-C virus.

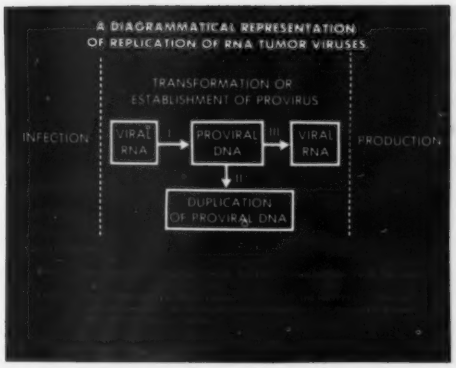

FIGURE 3.—Replication of type-C virus information and polymerases involved.

The viral RNA may act as a messenger to direct the synthesis of viral proteins (fig 3) (19,20). The viral RNA and proteins may then be assembled and released by budding from the cell membranes. These processes are poorly understood (1–3).

TYPE-C VIRAL-RELATED COMPONENTS IN HUMAN LEUKEMIA

New and more selective approaches to chemotherapy for neoplastic diseases will probably ultimately depend on our understanding of the molecular mechanisms involved in oncogenesis. We may assume on the basis of animal studies that the type-C RNA tumor viruses (or information related to these viruses such as "mis-evolution" of protoviruses as

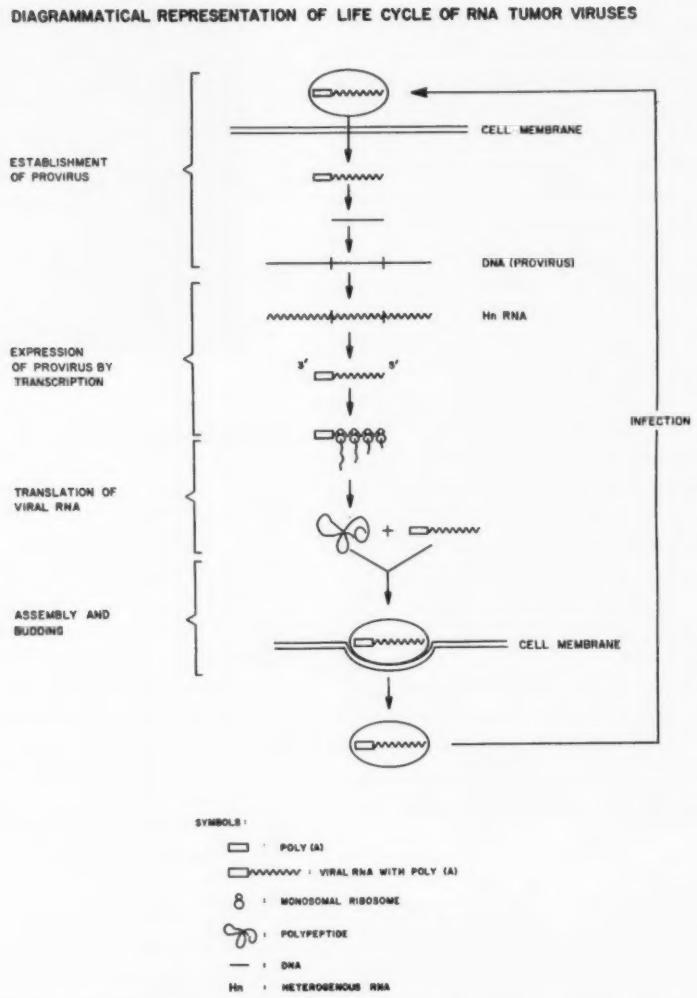

FIGURE 4.—Summary of some of the known molecular biologic events in the replication of type-C virus.

Temin says) will probably be important in man. However, before expending efforts to search for methods to interfere with their replication (or better the specific expression of this viral information in cells), ideally one would want to know if these viruses or components of these viruses are present in man. Are these the right viruses?

With the advances in the understanding of the molecular biology of these viruses and the increasing sophistication of the reagents used to study them, it is now clearly technologically possible to determine if these viruses or components of these viruses are present in man. This will not prove causality, but it is a critical first step, and a positive answer clearly indicates that attempts to understand their origin and their mechanism of transformation of cells by the use of animal models are of high priority.

Recent data have unequivocally demonstrated that type-C virus-related components are present in at least some human neoplasms. In human leukemic cells the evidence is best and is summarized here: (*a*) A DNA polymerase that could utilize both natural and synthetic RNA as template-primer from cells was first described by our laboratory specifically in human leukemic cells (21) after discovery of reverse transcriptase in extracellular RNA tumor viruses by Temin and by Baltimore (22). (*b*) Later, this activity was localized in cells to a cytoplasmic particle where it catalyzed an endogenous reverse transcriptase reaction (23–25). These cytoplasmic particulate fractions have a density similar to but often somewhat heavier than type-C virus. (*c*) These fractions can be converted to structures with the density of virus cores (Spiegelman S, personal communications). (*d*) The particles contain high molecular weight RNA—35S or 60S–70S (26,27). (*e*) DNA is made with sequences slightly related to mouse leukemia virus (26) but especially to primate tumorigenic (SiSV) and murine type-C sarcoma virus RNA (25). (*f*) Some of the nucleic acid sequences in leukemic cells appear to be unique, ie, not found in normal leukocytes (28). (*g*) The reverse transcriptase from this fraction (from acute myeloblastic leukemia cells) has been purified and shown to have biochemical (29,30) and (*h*) immunologic (in the case of acute myeloblastic leukemias) (27,31) properties closely related to reverse transcriptase from tumorigenic primate type-C viruses, ie, SiSV and GAL. With regard to the immunologic studies, it has been shown that purified antisera (IgG) prepared in rabbits or rats against purified reverse transcriptases from both SiSV and GAL (31) neutralize the human leukemic reverse transcriptase activity but do not inhibit the three major DNA polymerases from normal uninfected cells. Conversely, purified antisera (IgG) made against a major cellular DNA polymerase from normal human lymphocytes (DNA polymerase I) do not inhibit the human leukemic reverse transcriptase nor reverse transcriptase from animal viruses.[4]

[4] Smith RG, Lewis BJ, Abrell JW, et al. Manuscript submitted for publication.

In Vitro Assays for Early Prognosis and Diagnosis

When fully developed, human acute leukemia can be readily diagnosed by cytologic evaluation of the peripheral blood and bone marrow. However, when the number of leukemia cells is low, the diagnosis can be difficult or impossible to make due to the limited sensitivity and accuracy of current cytologic diagnostic methods. For years, the most critical level of sensitivity for the diagnosis of acute leukemia has been the finding of 5% or more morphologically identifiable blast or stem cells in the bone marrow, since this is the upper limit for this type of cell in normal bone marrow. Under these circumstances it is estimated that as many as 10^9 leukemic blast cells may be present in an affected individual (32). The clinical situations in which this diagnostic problem arises fall into two general categories: (*a*) the "preleukemic" state and (*b*) established disease after remission induction by chemotherapy. Recently, attempts have been made to more precisely define "preleukemia" from observations that certain disturbances in hematopoiesis, associated with abnormal bone marrow histology, are frequently followed by the development of frank leukemia (33,34). In terms of a diagnostic problem, we would also include in this category individuals who are in danger of developing leukemia because of predisposing genetic or environmental factors (35). In the remission state, examination for residual or recurrent disease is a routine procedure used for evaluating requirements for additional therapy. This diagnostic monitoring is frequently frustrated not only by limited sensitivity of cytologic techniques but by drug therapy-induced alterations in normal cells which make them more difficult to distinguish from leukemic cells.

Cell kinetic studies indicate that the primary defect in leukemia is mainly the result of a failure of white blood cell (wbc) precursors to properly differentiate (36,37). There are two possible explanations for this defect: (*a*) some extrinsic factor required for the differentiation of wbc is inactive or deficient, or (*b*) leukemic cells are intrinsically abnormal and cannot properly respond to stimuli for differentiation

(38). If the former is correct, the best hope for improved diagnostic sensitivity might be to detect abnormal differentiation factors or possibly excessive amounts of cell proteins which are characteristic of undifferentiated wbc. If, on the other hand, leukemic cells are intrinsically abnormal, there is the reasonable hope of developing sensitive and specific tests for detecting qualitatively different molecules not present in normal cells. However, it should be cautioned that intrinsic abnormalities could be due to disturbances in the regulation of gene expression and do not necessarily indicate the presence of structurally unique molecules. Although there is some evidence to support the extrinsic defect hypothesis (39), we feel that current data and knowledge are most consistent with a primary internal derangement of wbc precursors. For reasons discussed below, we are particularly concerned with the possibility that RNA tumor viruses may be involved in the cellular derangement, and we will present evidence which suggests that RNA tumor virus-related molecules in human leukemic cells may provide specific targets for appropriate diagnostic probes.

Both reverse transcriptase and 70S RNA have been found in human leukemic cells. An important characteristic of reverse transcriptase and 70S RNA in leukemic cytoplasmic particles is that they are physically complexed. This is demonstrated by their ability to "endogenously" synthesize DNA from deoxynucleoside precursors (4,26,29). This reaction is perhaps the most stringent criterion for a true reverse transcriptase; however, it requires careful analysis of the reaction products to be certain that endogenous, native RNA has been transcribed, and specifically that heteropolymeric and not just homopolymeric regions are transcribed (25,40). Two tests have frequently been utilized to further analyze this reaction. First, it may be shown that the reaction mixture with ribonuclease (24,25,30) is inhibited or abolished by pretreatment of the mixture. Although this is a useful screening procedure, it does not discriminate between the possibilities of destruction of RNA template or of RNA primer for a DNA-directed reaction (25,41). Second, since some of the DNA reaction product remains associated with the RNA template, it has been possible to detect RNA-DNA hybrids with the discrete size of viral RNA (26), which in our experience has been most frequently 35S (27).

To our knowledge, the endogenous DNA synthesis utilizing an RNA template (reverse transcription) has not been detected in any normal, fresh, adult mammalian tissue, proliferative or nonproliferative. We previously reported that a cytoplasmic pellet fraction from phytohemagglutinin (PHA)-stimulated normal human lymphocytes supported ribonuclease-sensitive endogenous DNA synthesis (41). Subsequently, we determined that this was primarily due to the presence of mitochondria in the pellet fraction and most of the reaction can be explained by an RNA-primed but DNA-templated reaction (42), although we have not ruled out an RNA-directed component. Kang and Temin have reported RNA→DNA systems in virus-free chicken embryos (43). This finding is not particularly surprising since RNA "tumor" viruses have been detected in embryonic or fetal tissues (including primate placenta; ref 6) and in a wide variety of mammalian tissue culture cells (none human or primate) before or after virus-induction treatment (44). Although these so-called endogenous viruses have the typical biochemical features of RNA "tumor" viruses, most may not be oncogenic. It is notable that Mizutani and Temin (45) determined that the endogenous DNA synthesis in the uninfected chick embryo was not strongly immunologically related to components of known avian tumor viruses.

These findings may have an important bearing on the utilization of virus-specific molecules in human leukemic cells as diagnostic markers. The reason for this is that hematopoietic stem cells, as immature, undifferentiated cells, are in a sense vestiges of embryonic cells, which are carried throughout life. Due to their unavailability in sufficient quantity for biochemical analysis, it is not presently known if they contain any RNA "tumor" virus components, although by analogy with known animal systems this would not be particularly surprising. This is one of the reasons that we

feel it is critical to develop diagnostic probes *specifically* related to *presumed* oncogenic viral components. Whether endogenous reverse transcriptase systems are present in hematopoietic stem cells and whether such components may have cross-homology with those in leukemic cells remain to be determined. If no cross-homology to virus-related components present in leukemic cells is found in mature or stem cells, then the molecular probes (described in the next sections) should provide specific reagents, which can be adopted to very sensitive detection methods for improving our diagnostic ability in leukemia and very likely other cancers.

Potential Diagnostic Application

The availability of specific reagents for virus-related molecules which may be unique to leukemic wbc greatly enhances the possibility of devising sensitive detection methods, which require smaller amounts of cells, for both analytic and clinical purposes. One possibility in this regard is to use sensitive immunologic methods, such as radioimmunoassay and immunoelectron microscopy, for detection purposes in disrupted cells. For reverse transcriptase, these methods can certainly be combined with biochemically monitored purification procedures, if necessary, to provide increased levels of sensitivity, or they may possibly be applied to the detection of other, non-enzymologic viral proteins.

An alternative use of virus-specific probes, which shows considerable promise, is the in situ detection of target molecules in nondisrupted, fixed-cell preparations. Not only would these techniques require much smaller amounts of cells, they can answer questions not easily approached by biochemical techniques using disrupted cells. For example, what is the intracellular location of virus-specific molecules? Is viral information contained in all leukemic cells or in larger amounts in but a few cells? The in situ mode may be adaptable either to the use of specific antisera or of virus-specific DNA probes; or, possibly, these two reagents might be used sequentially on the same cellular preparation. In this way, it is at least possible that the presence of viral genetic information, as determined by in situ nuclear DNA-DNA hybridization, could be correlated with the expression of viral information, as determined by the detection of antigen-antibody complexes in the cytoplasm. Although these techniques are in an early developmental stage, recent technical advances enhancing detection sensitivity make it probable, *if adequate specificity can be achieved,* that these measures will become available for analytic use and, possibly, for clinical application in the near future. They might then provide invaluable diagnostic and prognostic indicators by vastly improving the detection of malignant cells among a population of normal cells and perhaps by being able to detect signs of biochemical transformation before it is morphologically apparent.

In Vitro Systems for Studying Inhibitors of Type-C Virus Function

The rationale for developing inhibitors of viral replication has been previously described (46,47). It should be emphasized that the rationale is limited to *prophylactic* use and possibly to *remission maintenance.* There is as yet no logic for interfering with virus replication for treating cells already transformed. There are data from animal systems which indicate that continued *expression* of viral information is required to maintain cells in the transformed state (48). It may also be possible to test this in man by examining a wide number of neoplasms at various stages of the disease and correlating the presence of cytoplasmic viral RNA with the state of the disease. If there is good correlation, one may deduce that continued expression of viral information is also required to maintain the neoplastic state in man. If so, the logic would be to search for mechanisms to interfere with virus RNA formation. This may be very difficult in view of the data indicating that host cell mechanisms are probably utilized in the synthesis of viral RNA from viral proteins, but, on the other hand, viral RNA is larger than other cell messenger RNA (mRNA) and contains longer sequences of poly(A) than does cell cytoplasmic mRNA (49), suggesting that we may find something unique in its formation. Unfortunately, there are no specific rapid in vitro methods to evaluate compounds that may specifically interfere with this except to measure

inhibition of virus production in virus-producing cells, which, of course, may arise from many different effects having nothing to do with transformation. *The available studies on inhibitors of in vitro virus function have all been carried against virus replication.* The approaches used in our laboratory and some results are described below.

For convenience, viral replication has been roughly divided into three steps: infection, transformation, and production. The enzymes involved in some of these steps are indicated in Roman numerals in figure 3. As discussed earlier, the discovery of viral DNA polymerase (reverse transcriptase) supported the idea that the genetic information of RNA tumor viruses can be transmitted in a form of DNA termed "provirus," and it has been suggested on the basis of inhibition studies that reverse transcriptase is required for viral transformation in culture (14) and leukemic development in mice (15). The provirus can be transmitted to daughter cells at cell division. Presumably, its duplication requires DNA-dependent DNA polymerases of the host cell although a viral-specific DNA polymerase has not been excluded. After formation of the provirus, the genetic information of the RNA virus may be partially repressed (as in the case of producing cells). In some instances, a nonproducer can be induced to produce viruses by exposing the cells to chemicals such as iododeoxyuridine (IDU) (50). A simple model for virus production in cells containing proviral DNA is as follows: the RNA of RNA tumor viruses is synthesized via transcription of proviral DNA in a manner similar to that of cellular mRNA synthesis in that there is a posttranscriptional addition of poly(A) sequence to the selected mRNA. To accomplish this transcription, a cellular RNA polymerase and poly(A) synthetase are presumably required. The viral RNA, after processing, may then serve as messenger for protein synthesis. The same source of RNA is packaged into the viral particles and will serve as a template for reverse transcriptase upon further infection.

The enzymes required for the process of viral infection and viral replication may be useful targets in finding compounds potentially useful for antiviral chemotherapy. In order that the compounds can be biologically useful, they should have, along with their antienzymatic activity, the following two properties: (*a*) antiviral activity and (*b*) selective cytotoxicity to tumor cells.

STUDIES ON DERIVATIVES OF RIFAMYCIN THAT INHIBIT VIRAL REVERSE TRANSCRIPTASE

In a study of more than 200 rifamycin derivatives (51), we and others (52) found that some of these compounds (class A derivatives) are weak inhibitors of both viral reverse transcriptase and purified human DNA polymerases. Other compounds (class B derivatives) inhibit these enzymes with moderate potency. Still other compounds (class C derivatives) are highly potent inhibitors. Recent studies on the mechanism of action of the class C derivatives have demonstrated that they bind directly to reverse transcriptase (fig 5), that the enzyme inhibition depends on this binding (53), that the binding is reversible (53,54), that from two to 14 molecules of these compounds bind to each polymerase molecule, and that they act preferentially on the free form of the enzyme (53). The binding is not as "tight" as that to *Escherichia coli* RNA polymerase but interacts with these DNA polymerases more than with any nonpolymerase protein tested (53).

When murine tumor viruses are pretreated with these compounds, a direct correlation is observed between inhibition of focus formation or leukemogenicity and inhibition of reverse transcriptase of murine leukemia-sarcoma virus by class B and C derivatives (14,15). Table 1 gives examples of these results with two specific compounds. Attempts were then made to treat mice with these active compounds before and after infection with Rauscher leukemia virus (RLV). Only slight inhibition was observed when mice were treated prior to RLV infection even with a potent compound such as dimethylbenzyl-rifampicin [5] (table 1).

[5] Dimethylbenzyl-rifampicin: NSC-143414; CAS reg. No. 13292-47-2; 2,7-(epoxypentadedeca[1,11,13]trienimino)naphtho[2,1-*b*]-furan-1,11(2*H*)-dione, 8-[*N*-(4-

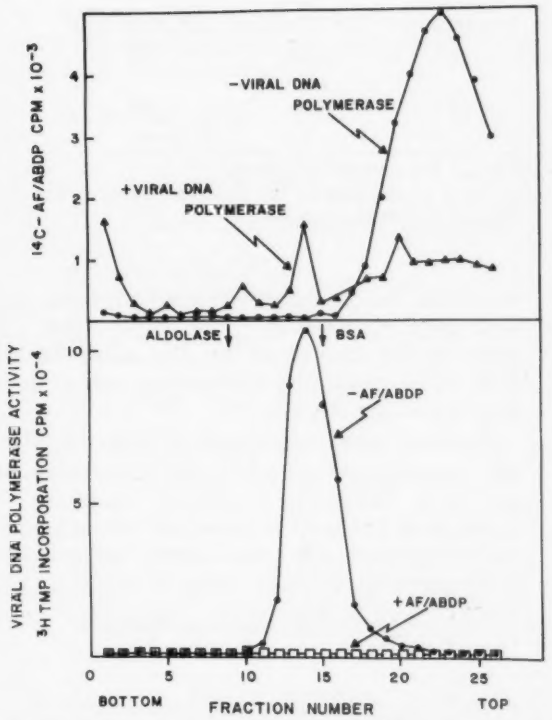

FIGURE 5.—Binding of dimethylbenzyl-rifampicin (AF/ABDP) to electrophoretically pure RLV reverse transcriptase. Glycerol velocity gradient sedimentation of enzyme alone (lower panel, –AF/ABDP, ●—●); enzyme with AF/ABDP (lower panel, + AF/ABDP, □—□); enzyme with ^{14}C-AF/ABDP (upper panel, + viral DNA polymerase, ▲—▲); and ^{14}C-AF/ABDP alone (upper panel, –viral DNA polymerase, ●—●). The results in the lower panel (enzyme activity) show that the pure enzyme moves as a discrete peak with an estimated size of about 70,000 daltons, and after preincubation with AF/ABDP no polymerase activity is detectable. The results in the upper panel (^{14}C-AF/ABDP) show that as expected, ^{14}C-AF/ABDP alone remains at the top of the gradient, but when ^{14}C-AF/ABDP is first preincubated with the purified polymerase a discrete peak is found (tubes 12–16) which corresponds to the polymerase activity peak. The details of this experiment have been presented elsewhere (53) as has the structure of the compound and its relative inhibitory activity (51).

TESTS ON PROPHYLACTIC ACTIVITY OF *N*-DEMETHYL-RIFAMPICIN ON AKR SPONTANEOUS LEUKEMIA

Since some rifampicin SV[6] derivatives have antiviral activity, we decided to test whether they may prevent spontaneous leukemia in AKR mice. These experiments were done by Dr. Lloyd Law of the National Cancer Institute. We chose *N*-demethyl-rifampicin[7] for these experiments because of its minimal cytotoxicity (46), although it is a weak (class B) reverse transcriptase inhibitor (14). Pregnant mice were inoculated (50 mg/kg) with this compound subcutaneously three times a week for a total of 3 weeks during mid-pregnancy. The newborns were also inoculated with the same dose during their first month of life. As shown in figure 6, the incidence of leukemia was delayed in the experimental group. Eventually, however, the experimental group reached the controls and leukemia ultimately developed in almost 100% of the animals. It will be of interest to determine if continued treatment would further delay or completely prevent the disease. Growth of the treated animals was not impaired, and there was no evidence of toxicity.

OTHER AGENTS THAT INHIBIT REVERSE TRANSCRIPTASE

A large number of agents other than rifampicin SV derivatives have been reported to inhibit reverse transcriptase. Examples of different classes of these are listed in figure 7. Perhaps the most extensively analyzed have been the streptovaricins, which were studied by Carter and Brockman et al (55,56), and modified nucleic acid polymers and other

benzyl-2,6-dimethyl-1-piperazinyl) formimidoyl]-5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-, 21-acetate, *cis*-.

[6] Rifampicin SV: NSC-133100; CAS reg. No. 6998-60-3; 2,7-(epoxypentadeca[1,11,13]trienimino) naphtho-[2,1-*b*]furan-1,11(2*H*)-dione, 5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-, 21-acetate.

[7] *N*-Demethyl-rifampicin: NSC-143416; CAS reg. No. 13292-45-0; 2,7(epoxypentadeca[1,11,13]trienimino)-naphtho[2,1-*b*]furan-1,11(2*H*)-dione, 5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-(*N*-1-piperazinylforminidoyl)-, 21-acetate.

TABLE 1.—Effect of rifamycin derivatives on RNA tumor virus activities (14,15)

Compound	Class	Inhibition (%)			
		Treatment before infection*			Treatment before and after infection†
		Focus forming units	Reverse transcriptase	Splenomegaly	Splenomegaly
N-Demethyl-rifampicin (AF/AP)‡	B	45	45	54	32
Dimethylbenzyl-rifampicin (AF/ABDP)‡	C	96	86	100	28

*The concentration of each compound was 100 μg/ml in the preincubation mixture.
†Mice received iv inoculation 1 day prior to RLV infection, 1 inoculation at the time of infection, and 1 inoculation the day after infection. The amount of compound was 500 μg/mouse.
‡Manufacturer's coded symbols for these compounds.

agents that interfere with template or substrate binding to polymerase, which were studied by Chandra et al (57–59) and by Muller et al (60). Although many of these have proven antitumor effects, we still lack clear strong enzyme specificity in vitro, so it is difficult to prove what the direct in vivo antitumor effects are due to.

COMPOUNDS THAT INHIBIT OR ENHANCE TRANSCRIPTION OF PROVIRUS

If expression of the provirus genome involves mechanisms similar to mechanisms involved in the transcription and processing of host mRNA, production of viral RNA will probably be influenced by the same factors that affect synthesis, processing, and translation of cellular mRNA.

To study gene expression of proviral DNA and compounds that affect this expression, we used the induction procedure described by Lowy et al (50). In this system virus production in murine cell lines can be achieved by induction with IDU of nonproducers (BALB/K3T3) and apparently uninfected cells (BALB/3T3).

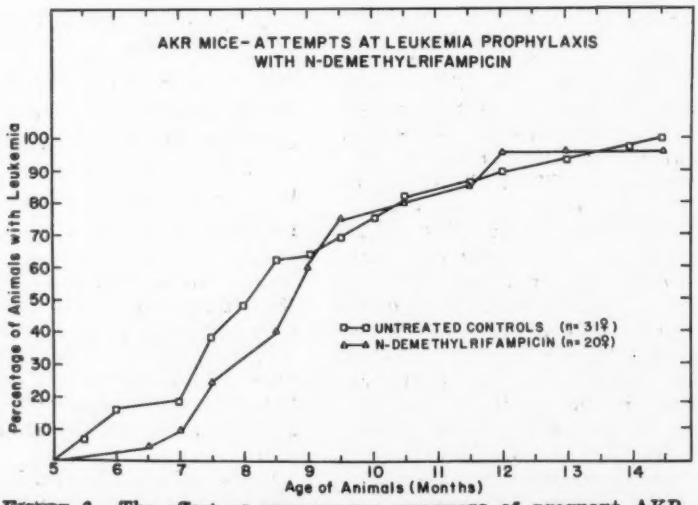

FIGURE 6.—The effect of propnylactic treatment of pregnant AKR mice and their newborn mice with *N*-demethyl-rifampicin on the incidence of spontaneous leukemia in these offspring. Experiments were carried out by Dr. L. Law. For details, *see* text.

I. Enzyme binding agents
   1. Ansa macrolides
      a. rifamycins
      b. streptovaricins
   2. Alkaloid extract of Narcissus tarzetta
   3. Pyran co-polymer

II. Substrate analogs
   1. Ara-CTP

III. Template-primer analogs
   1. $(A)n \cdot (dT)_{10} - R$
   2. Thymidylate derivatives

IV. Template "binding" agents
   1. Actinomycin D
   2. Chromomycin
   3. Daunorubicin
   4. Adriamycin
   5. Distamycin
   6. Ethidium bromide

V. Agents of unclassified mechanism of action
   1. Bleomycin
   2. Tilorone and tilorone cogeners

FIGURE 7.—Known inhibitors of reverse transcriptase. A more complete tabulation including references is presented in a brief review of reverse transcriptase inhibitors by Gallo and Smith (Life Sciences, in press).

Cordycepin, an inhibitor of poly(A) synthesis, has been reported to block synthesis of mRNA of adenoviruses (61). When this compound is added at the time of induction by IDU, production of type-C viruses is effectively blocked (18). The results of such an experiment are shown in figure 8 (upper panel). Maximum inhibition of induction was obtained when cordycepin was added simultaneously with IDU or within 24 hours after addition of IDU. The inhibitory effect decreased when cordycepin was given more than 24 hours after induction. The effective action of cordycepin requires its presence during a certain critical time interval, 12–18 hours after the addition of IDU. The mechanism of action of cordycepin on the inhibition of virus production is uncertain. Most likely, it acts to block poly(A) synthesis, but general inhibition of RNA or DNA synthesis cannot be ruled out.

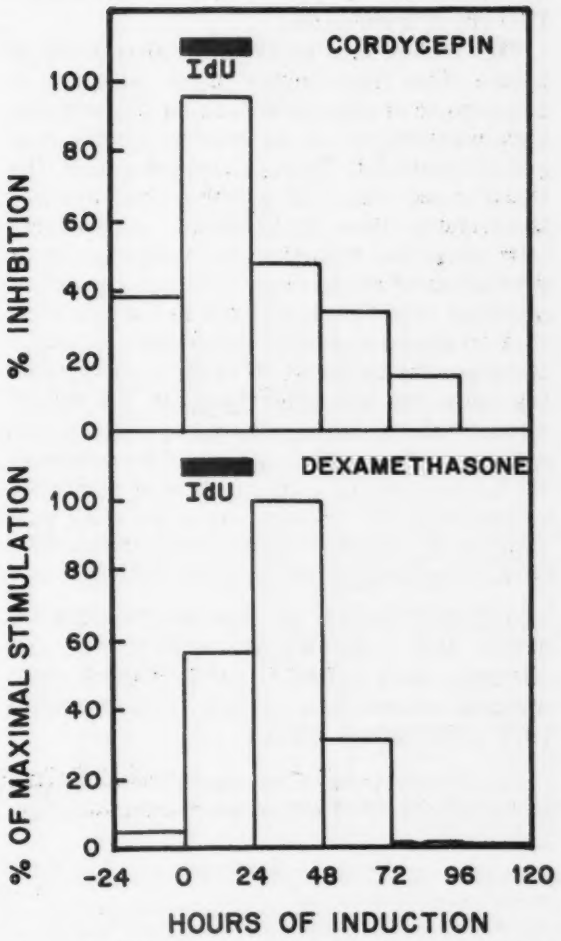

FIGURE 8.—Effect of time of addition of cordycepin and of dexamethasone on induction of virus production of BALB/K3T3 cells by IDU.

We have also used cordycepin to treat RLV-infected mice. A 50% reduction in spleen weight was observed without apparent toxicity with a treatment schedule of 10 mg/kg/day administered intraperitoneally for 4 days after inoculation of virus (62).

The other compounds we have tested for their effect on induction are the glucocorticoids (63). Although these hormones given alone did not induce virus production, they stimulated induction of virus by IDU five- to 25-fold. Apparently, this stimulatory activity is specific for glucogenic adrenal steroids. Maximum

stimulation of virus production occurs when dexamethasone is present 24–48 hours after IDU (fig 6, lower panel).

This finding may be useful in attempting to induce virus from human tumor cells, and it may also be of value in enhancing the immunologic reactivity of cancer cells containing viral genetic material. There is evidence that the transformed cells that produce virus are less tumorigenic than transformed nonproducer cells (64). The induction and enhancement of production of endogenous virus could result in a variety of antigenic changes in the cells such that an immunologically competent host might facilitate the rejection of these virus-producing cells. On the other hand, if the type-C viruses which were released contained the oncogenic information, this could be detrimental by contributing to reinfection of additional normal cells. We are now exploring these possibilities in leukemic mice treated successfully with chemotherapy but who later relapse.

TOXICITY STUDIES OF RIFAMYCIN DERIVATIVES AND ADRIAMYCIN (NSC-123127) ON NORMAL RAT KIDNEY (NRK) CELLS AND MURINE SARCOMA VIRUS-TRANSFORMED NRK (NRK[MSV]) CELLS

Some derivatives of rifamycin were reported to be selectively cytotoxic for human leukemia cells as compared to PHA-stimulated normal lymphocytes (65). This result is summarized in table 2. The question arises whether PHA-stimulated normal lymphocytes are the proper control cells for human leukemia cells. Animal tumor cell lines such as L1210 or L5178Y mouse leukemia cells have often been used to test toxicity of antitumor agents. Again there are no proper control cells for these tumor cell lines. An attempt was made, therefore, to select an animal cell system to test these positive compounds and to determine if an animal model in vitro system could predict for human tumor cell cytotoxicity. We chose NRK cells and NRK(MSV) cells because (*a*) they are of the same origin, and (*b*) both cell lines have a plating efficiency of 50%–60%, therefore a quantitative measurement can be obtained (62). Table 3 gives a summary of some preliminary results on the effect of some rifamycin derivatives and adriamycin, a known antitumor antibiotic, on colony formation of NRK and NRK(MSV) cells. In this system, a marginally selective toxicity is observed for the transformed cells, however, we have not tested enough compounds to adequately assess the system's effectiveness.

Based on new knowledge of mechanisms of viral oncogenesis, in vitro systems for assaying compounds for antiviral and antitumor ac-

TABLE 2.—Cytotoxicity of rifamycin derivatives for normal and leukemic human blast cells*

Compound tested	Blast cells (% of fraction in untreated culture)		
	PHA-stimulated normal blood lymphocytes	Leukemic lymphoblasts	Leukemic myeloblasts
Rifamycin SV	90 ± 10	42 ± 42	33
Dimethylbenzyl-rifampicin (AF/ABDP)	90 ± 10	47 ± 31	14 ± 10
3-Formyl-rifamycin SV: *O-n*-octyloxime (AF/013)	72 ± 4	7	0

*Normal and leukemic peripheral blood leukocytes were harvested from the blood of humans as previously described (65). Normal cells were stimulated with PHA for 48 hrs prior to this experiment (65). Cells were then seeded at a concentration of 10^5/ml in 6 ml of medium (McCoy's modified, 30% fetal calf serum) containing 50 μg/ml of derivative (added in 10 μl of DMSO) or 10 μl of DMSO (nontoxic at this concentration). After 48 hrs exposure to derivative (24 hrs in the case of the leukemic lymphoblasts), cells were harvested, fixed, and examined microscopically as previously described (65). Blast cells were first counted as a fraction of total leukocytes. This fraction was then expressed as a percentage of the blast cell fraction in control cultures (containing DMSO alone). Standard errors are included when more than 1 donor's cells were studied.

TABLE 3.—Effect of rifamycin derivatives and adriamycin on colony formation* of NRK cells and NRK(MSV) cells

Treatment	Survival (%)		Ratio: NRK(MSV)
	NRK	NRK(MSV)	NRK
None	100	100	1
Rifampicin (10 μg/ml)	95	61	0.64
Dimethylbenzyl-rifampicin (1 μg/ml)	85	50	0.59
Adriamycin			
0.01 μg/ml	54	29	0.54
0.005 μg/ml	87	50	0.57

*About 200 cells are seeded in 60-mm Falcon plastic plates in 3 ml medium (McCoy's 5A with 10% fetal calf serum). The next day, 3 ml of medium or medium containing a proper amount of the compound being tested is added to the appropriate plates. Cultures are fed with medium or medium containing tested compound on Days 4 and 7. No. of colonies are counted on Day 8.

tivity are available. The effectiveness of these systems in predicting accurately any antiviral antitumor activity in animal systems remains to be determined.

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Molecular Evidence for Viral Agents in Human Cancer and Its Chemotherapeutic Consequences [1]

S. Spiegelman [2]

SUMMARY

DNA-RNA hybridization was used to explore whether human neoplasias contain RNA molecules having sequence homologies to those of the RNA tumor viruses known to cause similar diseases in animals. The pattern of specific RNAs found in the human tumors showed a remarkable concordance with the predictions deducible from the animal systems. Thus, human breast cancer contains RNA homologous only to that of the murine mammary tumor virus. Human leukemias, sarcomas, and lymphomas (including Hodgkin's and Burkitt's lymphomas) all contain RNA with sequence homology to the murine leukemia virus and not to murine mammary tumor virus RNA. Finally, as in the case of the mouse, none of the human tumors examined contain RNA related in sequence to that of the avian myeloblastosis virus. The RNA detected in all of the human neoplasias was demonstrated to be of high molecular weight (1×10^7 daltons) and encapsulated with a reverse transcriptase in particles having densities between 1.16 and 1.19 g/ml. Further, the RNA of these human tumor particles was related in sequence to the murine viruses that cause the corresponding neoplasias in mice. Thus, four features diagnostic for the murine oncogenic viruses are satisfied by the particles found in the human cancers. Finally, it was shown by "recycling" experiments that the DNA from human leukemic cells and from lymphomatous tissue contained particle-related sequences that could not be detected in normal DNA. This finding was further substantiated by studies with identical twins in which it was shown that the leukemic twin contained particle-related sequences that could not be detected in the leukocytes of his identical healthy sibling. These findings are inconsistent with hypotheses that require chromosomal transmission in the germ line of complete copies of the information required to produce malignancy and the associated virus particles.

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[2] Institute of Cancer Research and the Department of Human Genetics and Development, College of Physicians & Surgeons, Columbia University, New York, NY.

It is obvious that the evolution of a rationale for the chemotherapy of cancer would be greatly aided by acquiring some insight into the etiology of this disease. If the conversion of a normal cell to a cancer cell involves the expression of viral or nonviral preexistent genetic information, then its control by exoge-

nous therapeutic agents will require a detailed chemical understanding of the key molecules involved, whether they be gene repressors or modulators of metabolic function in the cytoplasm or at the cell membrane. If, on the other hand, malignant transformation is preceded by the entry and insertion of new viral information, then these steps could provide additional and possibly more accessible targets for chemotherapy.

In approaching the problem of the etiology of human cancer we were unavoidably influenced by our previous experiences with RNA viruses and our assessment of the information available on the relationship between viruses and cancer. Both DNA and RNA viruses have been identified as capable of producing tumors in animals in laboratory experiments. Nevertheless, we decided to concentrate our experimental efforts on the RNA oncornaviruses, a choice made for two reasons. One is that the RNA oncogenic agents predominate quantitatively in the animal systems studied. The second reason is that it is the RNA tumor viruses that have been repeatedly demonstrated to cause tumors in their indigenous hosts. Thus, from the viewpoint of the natural history of the disease, the RNA oncogenic viruses appeared to us as the more probable "natural" candidates for etiologic agents of cancer in the animal kingdom, including man.

Over the past 50 years, animal viral oncology has amassed a considerable fund of information concerning the so-called B- and C-type RNA tumor viruses (1). These agents have been implicated in a variety of cancers in a wide spectrum of animals. In many instances, convincing evidence has been obtained by the inoculation of purified viruses into susceptible animals in experiments that satisfy Koch's criteria for the identification of the causative agent of a particular disease. Our laboratory has focused on an attempt to get an answer to the following question: "How much of this experience with animal viral oncology is transferable to human cancer?"

Our working assumption was based on the improbability that cancer in man would have so different an etiologic basis that the information provided by animal experiments would be irrelevant to the human disease. If this be the case, one might well wonder why it has been so difficult to provide acceptable evidence for a viral agent in human cancer. A plausible explanation emerges from an examination of the historical development of animal viral oncology. Forty to 50 years ago, it was as difficult to identify viruses in animal tumors as it is today in the human neoplasias. The experimental picture changed dramatically as the result of a decision with an interesting serendipic effect. A group of investigators, including Little, Strong, MacDowell, and Furth (cited in ref 1), developed highly inbred strains of mice, some of which were characterized by a high frequency of various neoplasias, including mammary tumors, leukemias, etc. The intent was of course to mate these neoplasias with low-frequency strains and from the distribution of the characteristic cancer phenotype deduce the genetic factors underlying susceptibility and resistance to the disease. What occurred unexpectedly, however, was that in the course of developing the inbred stocks of high cancer frequency, a genetic background was inadvertently created permitting the tumor viruses to multiply to levels that made their identification inevitable. This, along with the introduction in 1951 by Gross (1) of neonatal mice for virus assay, did much to open up the field of viral oncology.

Those who are concerned with human neoplasias are for the most part faced with the same difficulties encountered by the early animal oncologists when only outbred animals were available. It follows that the detection of putative human viral agents requires more sensitive devices than those which sufficed to establish their presence in the inbred animal systems. In searching for such tools we quite naturally turned to molecular hybridization and the other methodologies developed by molecular biologists in the past several decades.

Our investigations evolved through a number of stages that are conveniently identified by the questions we posed sequentially for experimental resolution as our studies progressed.

a. Do human neoplasias contain RNA molecules that possess detectable homology to the RNA of tumor viruses known to cause similar cancers in animals?

b. If a positive outcome is obtained, do the

RNA molecules identified in tumors possess the size and physical association with reverse transcriptase that characterize the RNA of the animal oncornaviruses?

c. If such RNA exists in human tumors, is it encapsulated in a particle possessing the density and size of the RNA tumor viruses?

d. Does the RNA of human tumor particles possess homology to the RNA of the viruses causing the corresponding disease in animals?

e. The virogene concept proposes that all animals prone to cancer carry in their *germ line* a complete copy of the information required to convert a cell from normal to malignant and for the production of tumor virus particles. Is this concept valid for randomly bred populations and in particular for the human disease?

The Animal Models as a Point of Departure

To better understand the biologic rationale underlying the experiments to be described, it is useful to summarize briefly some of the salient features of the animal model systems used as a guide. Table 1 lists a representative group of animal viruses, including those actually employed to generate the molecular probes used. There are two avian viruses, myeloblastosis virus (AMV) and Rous sarcoma virus (RSV), that cause leukemias and sarcomas in chickens. In addition, we have the murine leukemia virus (MuLV) and murine sarcoma virus (MuSV) that induce similar diseases in mice, and finally the murine mammary tumor virus (MMTV) that is the unique etiologic agent for mammary tumors.

When these viruses are examined for sequence homologies among their nucleic acids, a rather informative pattern emerges. It will be noted that the two chicken agents (AMV and RSV) have sequences in common but do not show detectable homology with any of the murine agents. Turning to the murine viruses, we find that the nucleic acids of the leukemia, lymphoma, and sarcoma agents show homology to one another but not to either of the two avian agents or to the MMTV. Finally, the MMTV has a singular sequence homologous only to itself.

It is important to understand that a plus sign (table 1) does not indicate identity but simply sufficient similarity to be detectable by the relaxed hybridization conditions used in these initial exploratory experiments. Similarly, a minus sign (table 1) does not imply a complete absence of sequence homology but rather that none was detected by the procedures employed.

TABLE 1.—Comparison of some representative oncornaviruses*

Virus	Indigenous host	Homology to†— AMV	RSV	MuLV	MSV	MMTV	Disease
AMV	Chicken	+	+	-	-	-	Leukemia
RSV(RAV)	Chicken	+	+	-	-	-	Sarcoma
MuLV	Mouse	-	-	+	+	-	Leukemia, lymphoma
MSV(MuLV)	Mouse	-	-	+	+	-	Sarcoma
MMTV	Mouse	-	-	-	-	+	Breast cancer

*AMV = avian myeloblastosis virus; RSV = Rous sarcoma virus; RAV = Rous avian virus; MuLV = murine leukemia virus; MSV = murine sarcoma virus; MMTV = murine mammary tumor virus.

†The results of molecular hybridizations between [^{3}H]DNA complementary to the various RNAs and the indicated RNAs. + = Hybridizations were positive; - = hybridizations were not detected.

If analogous virus particles are associated with the corresponding human diseases, certain predictions might be hazarded on the basis of the specificity patterns exhibited in table 1, and these may be listed as follows:

a. in view of the lack of homology between the avian and murine agents it is unlikely that human agents (should they exist) would show homology to the avian group;

b. it follows that the murine tumor viruses would represent a more hopeful source of the molecular probes needed to search for similar information in the analogous human cancers;

c. if particles are found to be associated with human leukemias, sarcomas, and lymphomas, their RNAs might show homology to one another and possibly to that of the MuLV;

d. if RNA particles are identified in human breast cancer, they should not exhibit homology to the RNA of the viruses causing the human mesenchymal neoplasias or to RLV RNA, but might exhibit some homology to the RNA of the MMTV.

On the basis of the availability and the considerations outlined above, it is clear why the murine agents were initially chosen to produce the necessary molecular probes to look for corresponding information in the human disease. Furthermore, the desire to monitor the biologic consistency of our findings dictated that we examine a number of human neoplasias simultaneously. This would permit us to determine quickly whether our findings in man mirrored biologically what was known from the animal experimental models. For this purpose, from the onset we focused our attention in parallel on the mesenchymal neoplasias and on breast cancer.

Molecular Hybridization With Radioactive DNA Probes

To answer the first question posed on whether or not human tumors contain viral-related RNAs, we naturally turned to the use of DNA-RNA hybridization (2), which we designed some dozen years ago to answer questions of almost precisely this nature in the case of virus-infected bacteria. This method depends on the ability of any piece of single-stranded DNA to find its complementary RNA and form (under the proper conditions) a double-stranded hybrid structure. The reaction is highly specific and has proved to be of considerable value in molecular biology over the past decade.

The radioactive DNA needed was synthesized by supplying detergent-disrupted virus preparations with magnesium and the four deoxyribosidetriphosphates required, one of them being labeled with tritium. When the synthesis is terminated, the protein and the RNA present are eliminated and the residual radioactive DNA is purified to completion. Each [^{3}H]DNA preparation must be rigorously monitored for the specificity of its hybridizability to its appropriate template. Thus, each probe is challenged with its homologous RNA as well as with the other unrelated viral RNAs. Further, normal and cancerous tissue RNAs from animal models were used to check the reliability of the hybridizations. Only if a probe demonstrated the required specificity in these test hybridizations was it used for subsequent experiments with human material.

There are certain features built into these exploratory experiments that are worthy of explicit mention. We of course did not know initially if there would be *any* homology between the murine viruses and any putative human agents. At the time, RLV and MMTV were among the few mammalian oncornaviruses available in adequate supply and we could only hope that they would have sufficient homology to be used for our purpose. However, even if this hope could be realized, it would hardly be likely that the homology would be extensive. Therefore, hybridization experiments had to be carried out under conditions sufficiently relaxed to yield a reaction with a limited level of homology. Further, the method of detection of hybrid structures would have to possess a low background noise level so that weak positive reactions would be readily identified and distinguished from negative outcomes. Finally, the $C_{r_o}t$ (concentration of ribonucleotides in moles per liter × time in seconds) values of the annealing reactions

were deliberately adjusted to be adequate for multiple (ten or more) rather than individual copies per cell. This was done in order to magnify any existent difference between normal and malignant tissue. Initially, we were not concerned with determining whether normal cells could produce the same or related malignant messages at very low levels.

All of these considerations entered into the design of the detailed methodology used that is diagrammed in figure 1. After monitoring for specificity, the purified viral-specific [^{3}H]-DNA is mixed with cytoplasmic RNA prepared from a variety of human tumors and annealed under the conditions indicated. The hybridizations were always carried out with a vast excess of tumor RNA. Since the viral-specific [^{3}H]DNA is small compared with the RNA, any complexes formed between them will behave physically more like RNA than like DNA. Such complexes are readily detected by isopycnic separation in equilibrium density gradients of Cs_2SO_4. At the end of the centrifugation, the distribution of [^{3}H]DNA is examined across the gradient. Any uncomplexed DNA will remain at a density corresponding to about 1.45. DNA molecules that have annealed partially or completely to large RNA molecules will band at or near the density of RNA (ρ = 1.65).

The movement of the [^{3}H]DNA to the RNA region is then the signal that the probe used has found complementary sequences in the tumor RNA with which it is being challenged. It is important to emphasize that this test is essentially a qualitative one and does not measure how good the fit is in the duplex. A positive outcome says only that sufficient homology exists between the tritiated DNA probe and some RNA molecules in the tumor RNA sample to form a complex stable enough to be detected by the test used. Once such structures are identified, the fidelity of the pairing can be examined by a temperature-melt analysis. These more sophisticated examinations demanded more hybrids than we could initially produce. It was therefore necessary to postpone these more informative experiments until more efficient probes became available.

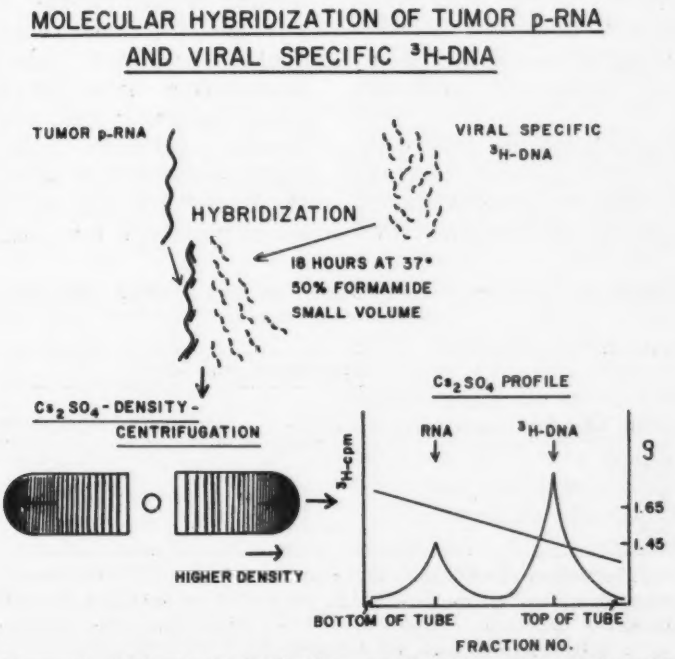

FIGURE 1.—Molecular hybridization and detection with viral-specific [^{3}H]DNA and tumor RNA (*see* text for further details).

We understandably began by exploring those human malignancies for which there were suitable animal models and viruses. These included adenocarcinoma of the breast (3,4), the leukemias (5), the sarcomas (6), and the lymphomas (7). The leukemias examined encompassed both acute and chronic varieties of the lymphatic and myelogenous types. The human sarcomas studied included fibrosarcomas, osteogenic sarcomas, and liposarcomas. The series on lymphomas contained Hodgkin's disease, lymphosarcomas, and reticulum-cell sarcomas. Control adult and fetal tissues were always examined in parallel and were invariably negative. In the case of breast cancer, the two benign diseases, fibroadenoma and fibrocystic disease, were also included and found to be negative.

Table 2 summarizes in diagrammatic form the outcome of this survey of human neoplasias with the animal virus probes. The plus signs indicate that the corresponding [^{3}H]DNA formed complexes with the indicated tumor RNAs and the minus signs indicate that no such complexes were detected. The proportion of positives among those complexes labeled plus in earlier studies (3–7) ranged from about 67% for breast cancer to 92% for the leukemias. As our technology improved so did our percent of positives among the neoplastic samples.

What is most noteworthy of the pattern exhibited in table 2 is its concordance with the predictions deducible from the murine system. Thus, human breast cancer contains RNA homologous only to that of the MMTV. Human leukemias, sarcomas, and lymphomas all contain RNA showing sufficient homology to that of the murine Rauscher leukemia virus (RLV) to make a stable duplex. These mesenchymal neoplasias contain no RNA homologous to the MMTV RNA. Finally, none of the human tumors contain RNA detectably related to that of the AMV. Recently, Gallo and his associates (8) have confirmed the homology of leukemic RNA to that of RLV and in the process have shown even more relatedness to the RNA of a simian sarcoma virus. In summary, the specificity pattern of the unique RNA found in the human neoplasias is in complete agreement with what has been described for the corresponding viral-induced malignancies in the mouse.

Etiology of Burkitt's Lymphomas

The discovery of RNA homologous to that of the MuLV in such human lymphomas as Hodgkin's disease, lymphosarcoma, and reticulum-cell sarcoma immediately raises the issue of Burkitt's disease, a malignant lymphoma found in children living in a geographical belt extending across central Africa. There exists serologic (9) and nucleic acid hybridization (10) evidence that links this disease to the Epstein-Barr virus (EBV), a DNA herpes-like agent detected in (11) and isolated from (12) Burkitt's lymphoma cells grown in culture. Since a causal relationship between EBV and the Burkitt's tumor has not been conclusively established, it is pertinent to inquire whether

TABLE 2.—Homologies among human neoplastic RNAs and animal tumor viral RNAs*

	Human neoplastic RNAs			
Viral RNAs†	Breast cancer	Leukemia	Sarcoma	Lymphoma
MMTV	+	–	–	–
RLV	–	+	+	+
AMV	–	–	–	–

*The results of molecular hybridization between [^{3}H]DNA complementary to the various viral RNAs and pRNA preparations from the indicated neoplastic tissues. + = Hybridizations were positive; – = hybridizations were not detected (7).

†MMTV = murine mammary tumor virus; RLV = murine Rauscher leukemia virus; AMV = avian myeloblastosis virus.

this lymphoma, like the others examined, contains RLV-related RNA.

In figure 2 we summarize and compare the results (13,14) of hybridizations with RLV-DNA probes and the cytoplasmic RNAs of Burkitt's tumors, Hodgkin's disease, and other human lymphomas. An examination of 21 African Burkitt's lymphomas clearly demonstrates that 76% of these tumors contained RNA related in sequence to that of RLV but not to that of MMTV or AMV. Further, as may be seen in figure 2, the pattern of specific RNAs contained in Burkitt's tumors was not distinguishable from that obtained in the other lymphomas. It can be noted that the cytoplasmic RNA extracted from a variety of 51 normal human adult and fetal tissues, including African nonneoplastic biopsies, and EBV-containing cell lines did not show annealing to RLV-DNA probes. These results raise obvious etiologic questions concerning Burkitt's lymphoma, to which we shall return.

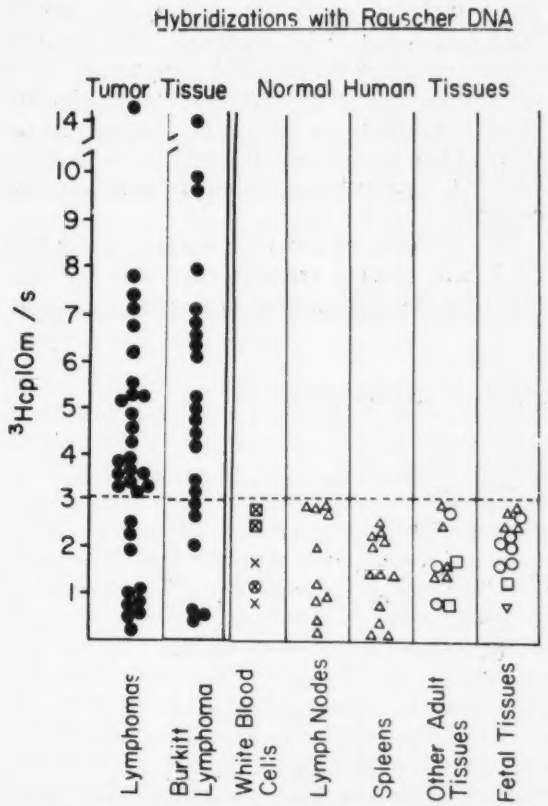

FIGURE 2.—Results of hybridization reactions with RLV [^{3}H]DNA and pRNA from human lymphomas, Burkitt's tumors, and normal human cells. The normal tissues tested are: normal wbc's (×); PHA-stimulated lymphocytes (⊠); human lymphocyte cell line NC-37 (⊗); lymph nodes, spleens, and other adult tissues (including liver [△], intestine [○], and striated muscle [□]); and fetal tissues (including liver [△], lung [▽], limbs [○], and placenta [□]).

Simultaneous Detection Test for Reverse Transcriptase and High Molecular Weight RNA

Needless to say, the existence of the remarkable concordance exhibited in table 2 does not establish a viral etiology for these diseases in man. The next step required the performance of experiments designed to answer the second and third questions raised in the introduction, ie, those relating to the size of the RNA being detected and whether or not it is associated with a reverse transcriptase in a particle possessing other features of complete or incomplete virus particles. We now turn our attention to the description of these techniques and their application to the analysis of human malignant tissue.

What we sought was a method of detecting the presence of particles similar to the RNA tumor viruses in human material that would be simple, sensitive, and sufficiently discriminating, so that a positive outcome could be taken as an acceptable signal for the presence of a virus-like agent. To achieve this goal, we devised a test that depended on the simultaneous detection of two diagnostic features of the animal RNA tumor viruses.

The oncornaviruses exhibit two identifying characteristics. They contain a large (1×10^7 daltons in molecular weight) single-stranded RNA molecule having a sedimentation coefficient of 60S–70S, often referred to as high molecular weight RNA. They also have reverse transcriptase (15,16), an enzyme that can use the viral RNA as a template to make a complementary DNA copy (17).

The possibility of a concomitant test for both the enzyme and its template was suggested by studies of the early reaction intermediates (17,18). The initial DNA product was found complexed to the large 70S RNA template. These structures can be detected by the unusual position of the newly synthesized small trit-

iated DNA products in Cs_2SO_4 density gradients, glycerol velocity gradients, and acrylamide gel electrophoresis (19). The most informative assay is to subject the isotopically labeled product to sedimentation analysis prior to the removal of the RNA. If the labeled DNA product behaves as if it is a 70S molecule, and if it can be shown that it does so because it is complexed to a 70S RNA molecule, then evidence is provided for the presence of a reverse transcriptase using a 70S RNA template. One may then tentatively conclude that the material examined contains particles similar to the RNA tumor viruses.

It was on this basis that Schlom and Spiegelman (20,21) developed the simultaneous detection test that was used to demonstrate (22) the presence in human milk of particles containing 70S RNA and a reverse transcriptase. The test was modified (23) to be applicable to cancerous tissue using the MMTV as the experimental model.

Figure 3 diagrams the procedure used. Tumor cells are first broken down by the use of a Dounce homogenizer and the nuclei and large-cell debri are removed by low-speed centrifugation. The supernate is subjected to trypsin digestion to inactivate any nucleolytic enzymes and the trypsin is neutralized by a trypsin inhibitor. The supernate is then centrifuged at 150,000 × *g* to yield a cytoplasmic pellet containing virus particles, if present. The pellet is then treated with a nonionic detergent (0.1% of NP–40) to disrupt possible viral particles and used in an endogenous reverse transcriptase reaction. It should be noted that detergent disruption is essential for a positive reaction, indicating that the enzyme and template are enclosed in a structure that must be opened up to make them accessible to the substrates. Actinomycin D (NSC–3053) is always included (100–200 μg/ml) to inhibit DNA-instructed DNA synthesis. The product of the reaction with its RNA template is freed of protein and then analyzed in a glycerol velocity gradient to determine the sedimentation coefficient of the [^{3}H]DNA as well as in a Cs_2SO_4 equilibrium gradient to determine its density.

The presence of particles encapsulating 70S RNA and reverse transcriptase will be indicated by the appearance of a peak of newly

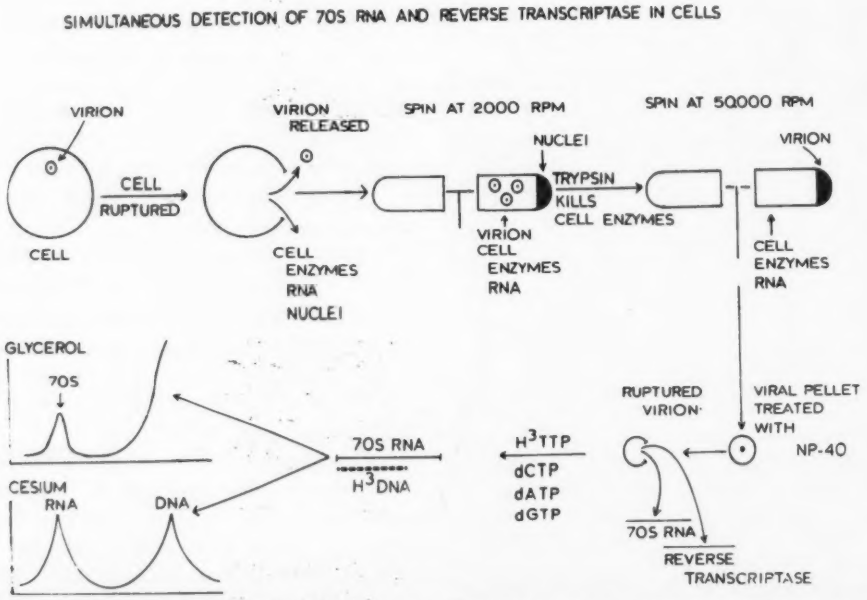

FIGURE 3.—Simultaneous detection test for 70S RNA and reverse transcriptase in neoplastic tissue (*see* text for further details).

synthesized DNA traveling at a speed corresponding to a 70S RNA molecule. That its apparent large size is due to its being complexed to a 70S RNA molecule can be readily verified by subjecting the purified nucleic acid to ribonuclease prior to velocity examination, and this should result in the disappearance of the "70S" [^{3}H]DNA. Similarly, if the reaction is positive, newly synthesized DNA should appear in the RNA region of a Cs_2SO_4 density gradient, and this again should be eliminated by prior treatment with ribonuclease.

The simultaneous detection test was first applied to human breast cancer (24) and it was found that 74% of 38 adenocarcinomas tested were positive for the simultaneous reaction, whereas ten nonmalignant controls including both normal tissues and benign tumors were all negative. It was further demonstrated (24) that the adenocarcinoma particles encapsulating the 70S RNA and reverse transcriptase possess a density between 1.16 and 1.19 g/ml, the density characteristic of the oncogenic viruses.

Application of the Simultaneous Detection Test to the Leukemias

In the study of the leukemias (25) peripheral leukocytes were prepared from the buffy coats of both leukemic and nonleukemic control patients. Cells were disrupted and fractionated as described in figure 3. Representative experiments examining the effects of the ribonuclease treatment of the product and omission of one of the deoxytriphosphates during the reaction are shown in figure 4. We see the telltale 70S peaks of DNA synthesized by the pellet fractions from the leukocytes of patients with acute lymphoblastic and acute myelogenous leukemias. The elimination of the complex by prior treatment with ribonuclease (fig 4*A*) shows that the tritiated DNA is indeed complexed to a 70S RNA molecule. Further, the omission of dATP (fig 4*B*) leads to a failure to form the 70S complex, a result expected if the reaction is in fact leading to the synthesis of a proper heteropolymer. In similar experiments, it was shown that omission of either dCTP or dGTP also resulted in the absence of the 70S RNA-[^{3}H]DNA complex, all of which argues against nontemplated end addition reactions.

In some cases leukemic cells were obtained in amounts adequate to permit a more complete characterization of the product. Hybridization of the human product to the appropriate viral RNAs provides the most revealing information since it tests sequence relatedness to known oncogenic agents. We summarize in table 3 the results of examining the peripheral leukocytes of 23 patients all in the active phases of their disease including both acute and chronic leukemias. Of the 23 leukemic patients examined, 22 patients showed clear evidence that their peripheral leukocytes contained particles mediating a reaction leading to the appearance of endogenously synthesized DNA in the 70S region of a glycerol gradient. Nine of these patients were tested for ribonuclease sensitivity and in all cases the complexes were destroyed. In nine other patients, the DNA was recovered from the complex and annealed to RLV RNA and to either MMTV RNA or AMV RNA, and in all nine, hybridizations occurred with RLV RNA and not to either of the unrelated MMTV RNA or AMV RNA. In four patients, enough DNA complex was formed to permit a complete characterization of the product, and in all four, the DNA complexes were destroyed by ribonuclease and the purified DNA hybridized uniquely to RLV RNA.

It is noteworthy that positive outcomes were observed in more than 95% of the leukemic patients whether they were acute or chronic, lymphoblastic or myelogenous. Thus, despite their disparate clinical pictures and differing cellular pathologies, these various types of leukemias have similar, though probably not identical, virus-related information.

In contrast with these results are those results obtained with a control series of 18 white blood cell (wbc) samples from nonleukemic patients. These included nine patients with elevated wbc counts (in the range of 25,000 cells/mm^3) due to a variety of disorders, including polycythemia vera. None of these exhibited evidence of an RNA-DNA complex that was ribonuclease-sensitive and contained DNA that hybridized specifically with RLV RNA. Thus, none of the 18 nonleukemic individuals ex-

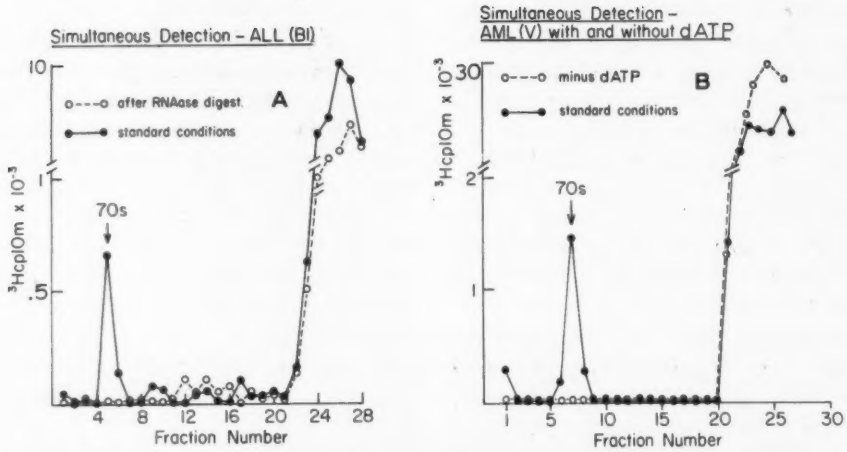

FIGURE 4.—Detection of 70S RNA-[³H]DNA complex in human leukemic cells. One g of leukemic wbc was washed in 5 ml of 0.01 M NaCl and 0.01 M tris-HCl (pH 7.4); resuspended in 4 ml of 5% sucrose, 0.005 M EDTA, and 0.01 M tris-HCl (pH 8.3); and ruptured with 3 strokes of a Dounce homogenizer. The nuclei were removed by low-speed centrifugation (2000 × *g*, 5 min, 2 C). The supernatant was brought to a final concentration of 1 mg/ml trypsin (Worthington) and incubated at 37 C for 30 min. A tenfold excess of lima bean trypsin inhibitor (Worthington) was added (final concentration, 3 mg/ml) and the solution again centrifuged at 2000 × *g* for 5 min at 2 C. The supernatant was then centrifuged at 45,000 rpm for 60 min at 2 C. The resulting cytoplasmic pellet was resuspended in 0.5 ml of 0.01 M tris-HCl (pH 8.3), brought to 0.1% Nonidet P–40 (Shell Chemical Co.), and incubated at 0 C for 15 min. DNA was synthesized in a typical reverse transcriptase reaction mixture (final volume, 1 ml) containing: 50 μM of tris-HCl (pH 8.3), 20 μM of NaCl, 6 μM of $MgCl_2$, 100 μM each of dATP, dGTP, and dCTP, and 50 μM of [³H]dTTP (Schwarz Biochemical, 800 cpm/pM). 50 μg/ml of actinomycin D was added to inhibit DNA-instructed DNA synthesis. After incubation at 37 C for 15 min, the reaction was adjusted to 0.2 M NaCl and 1% SDS, and was deproteinized by phenol-cresol extraction. The aqueous phase was layered on a 10%–30% gradient of glycerol in TNE buffer (0.01 M tris-HCl [pH 8.3], 0.1 M NaCl, 0.003 M EDTA) and centrifuged in an SW-41 rotor Spinco at 40,000 rpm for 180 min at 2 C. Fractions were collected from below and assayed for trichloroacetic acid-precipitable radioactivity. In this, as in all sedimentation analyses, 70S RNA of the AMV was used as a marker. *A*. One aliquot of product was run on the gradient as a control and the other was pretreated with 20 μg of RNase 1 (Worthington) for 15 min at 37 C prior to sedimentation analysis. *B*. Reactions with and without dATP (25).

hibited evidence of the specific 70S RNA-directed DNA synthesis found in virtually all of the leukemic patients examined.

Application of the Simultaneous Detection Test to Human Lymphomas

The human lymphomas previously studied by hybridization (7,13,14) were also examined (14,26) by simultaneous detection for particles containing reverse transcriptase and a 70S RNA template. The results obtained are summarized in table 4. It will be noted that 70% of the Hodgkin's diseases, 82% of the Burkitt's tumors, and 88% of the other lymphomas, including lymphosarcomas and reticulum-cell sarcomas, showed clear evidence of particles possessing features characteristic of the RNA tumor viruses.

TABLE 3.—Simultaneous detection of 70S RNA and reverse transcriptase in leukemic cells*

Patient No.	Disease	Simultaneous detection (cpm)	RNase sensitivity	Hybridization to RLV RNA	Hybridization to AMV RNA or MMTV RNA
1	Acute lymphatic leukemia	400	+	NT	NT
2	Acute lymphatic leukemia	95	+	NT	NT
3	Acute lymphatic leukemia/ lymphosarcoma	805	+	+	-
4	Acute lymphatic leukemia/ lymphosarcoma	200	NT	+	-
5	Acute lymphatic leukemia/ lymphosarcoma	185	+	NT	NT
6	Acute lymphatic leukemia/ lymphosarcoma	105	+	NT	NT
7	Acute myelogenous leukemia	170	+	NT	NT
8	Acute myelogenous leukemia	985	+	+	-
9	Acute myelogenous leukemia	305	+	NT	NT
10	Acute myelogenous leukemia	1295	+	+	-
11	Acute myelogenous leukemia	1010	NT	+	-
12	Acute myelogenous leukemia	115	NT	+	-
13	Acute myelogenous leukemia	415	NT	+	-
14	Acute myelogenous leukemia	400	NT	+	-
15	Acute myelogenous leukemia	605	+	NT	NT
16	Acute myelogenous leukemia	215	+	+	-
17	Acute myelogenous leukemia	285	+	NT	NT
18	Acute myelogenous leukemia	0	NT	NT	NT
19	Acute myelogenous leukemia	1400	NT	+	-
20	Chronic lymphatic leukemia	200	+	NT	NT
21	Chronic myelogenous leukemia	405	NT	+	-
22	Chronic myelogenous leukemia	390	NT	+	-
23	Chronic myelogenous leukemia	600	NT	+	-

*NT = not tested; RLV = murine leukemia virus; AMV = avian myeloblastosis virus; MMTV = murine mammary tumor virus.

TABLE 4.—Simultaneous detection tests with human lymphomas and controls*

Tissue	No. of positive reactions	Average "70S" (cpm)	No. of negative reactions	Average "70S" (cpm)	Positive reaction (%)
Hodgkin's disease	22	379	6	14	79
Burkitt's lymphoma	9	369	2	14	82
Other lymphomas	7	347	1	24	88
Control (spleens)	0	0	14	14	0

*Results of simultaneous detection assays with human lymphomas and controls. The average of the cpm in the 70S position monitored by external size markers is summarized. The reactions were designated as positive if the cpm exceeded 30 above background (14,26).

Implications of Simultaneous Detection Tests on Human Neoplasias

The experiments we have just summarized on human breast cancer, the leukemias, and the lymphomas were designed to probe further the etiologic significance of our exploratory in-

vestigations (3,5–7) which identified in these neoplasias RNA that was homologous to those of the corresponding murine oncornaviruses. The data obtained with the simultaneous detection test established that at least a portion of the tumor-specific virus-related RNA we were detecting was a 70S RNA template physically associated with a reverse transcriptase in a particle possessing a density between 1.16 and 1.19 g/ml, three of the diagnostic features of the animal RNA tumor viruses. Further, the DNA synthesized in the particles found in each type of neoplasia hybridized uniquely to the RNA of the corresponding animal oncornavirus. Note that this last result is complementary to and completes the logic of our experimental approach. We started out by using animal tumor viruses to generate [^{3}H]DNA probes that were used to find related RNA in human neoplastic tissue. We concluded by using analogous human particles to generate [^{3}H]DNA probes that were then used to determine sequence relatedness to the RNA of the relevant oncornaviruses. None of the human probes hybridized to the avian viral RNA. The probe generated by the particles from human breast cancer was homologous only to the RNA of MMTV, whereas the human leukemic and lymphoma probes were related in sequence only to RLV RNA, the murine leukemic agent. The biologic logical consistency of these results adds further weight to their relevance to human disease.

On the Problem of Germ-Line Transmission of Viral Information

We now come to grips with the popular virogene (27) concept that derives from animal experiments and argues that all animals prone to cancer contain in their germ line at least one complete copy of the information necessary and sufficient to convert a cell from normal to malignant and produce the corresponding tumor virus. This hypothesis presumes that the malignant segment usually remains silent and that its activation by intrinsic or extrinsic factors leads to the appearance of virus and the onset of cancer.

There are various ways of testing the validity of the virogene hypothesis, but the pathways differ in the technical complexities entailed. One approach commonly used attempts to answer the question: "Does every normal cell contain at least one complete copy of the required viral-related malignant information?" The methodologies employed included the techniques of genetics, chemical-viral induction, and molecular hybridizations. However, for a variety of reasons, none of these gave, or could give, conclusive answers. Genetic experiments do not readily distinguish between susceptible genes and actual viral information. Further, even if genetic data succeeded in identifying *some* structural viral genes, it would still be necessary to establish that *all* the viral genes are represented in the genome. Attempts to settle the question by demonstrating that *every* cell of an animal can be chemically induced to produce viruses have thus far (for obvious reasons) not been tried. The best that has been achieved along these lines was to show that *cloned* cells do respond positively. However, the proportion of clonable cells is small and *clonability may well be a signal for prior infection with a tumor virus.*

Finally, the quantitative limitations of molecular hybridization make it almost impossible to provide definitive proof that each cell contains one complete viral copy in its DNA. Although it is not very difficult to show that 90% of the information is present, it is the last 10% that constitutes the insurmountable barrier and 10% of 10^7 daltons amounts to a far from trivial 10^6 daltons, the equivalent of about three genes.

A useful way to obviate these technical difficulties is to invert the problem. Instead of asking whether one complete copy exists in normal cells, the question can be phrased in the following terms: "Does the DNA of a malignant cell contain viral-related sequences that are *not* found in the DNA of its normal counterpart?" Phrasing the issue in this manner leads to the design of experiments that avoid the uncertainties generated by the demonstrated fact that many indigenous RNA tumor viruses share (completely or partially) *some* sequences with the normal DNA of their natural hosts (28). The crucial point is of course whether *all* of the viral sequences are

to be found in normal DNA. The approach we adopted requires the removal of those viral sequences that are contained in nonneoplastic DNA by exhaustive hybridization of the viral probe to normal DNA in vast excess. Any unhybridized residue can then be used to determine whether malignant DNA contains viral-related sequences not detectable in normal tissue.

We first investigated this question in the case of the human leukemias (29) and the strategy, as diagrammed in figure 5 (*A* and *B*), may be outlined in the following steps:

a. isolate from leukemic cells the fraction enriched for the particles encapsulating the 70S RNA and RNA-directed DNA polymerase;

b. use this fraction to generate [^{3}H]DNA endogenously synthesized in the presence of high concentrations of actinomycin D to inhibit host and viral DNA-directed DNA synthesis;

c. purify the [^{3}H]DNA by hydroxylapatite and Sephadex chromatography, with care being exercised to remove all self-complementary material in the tritiated

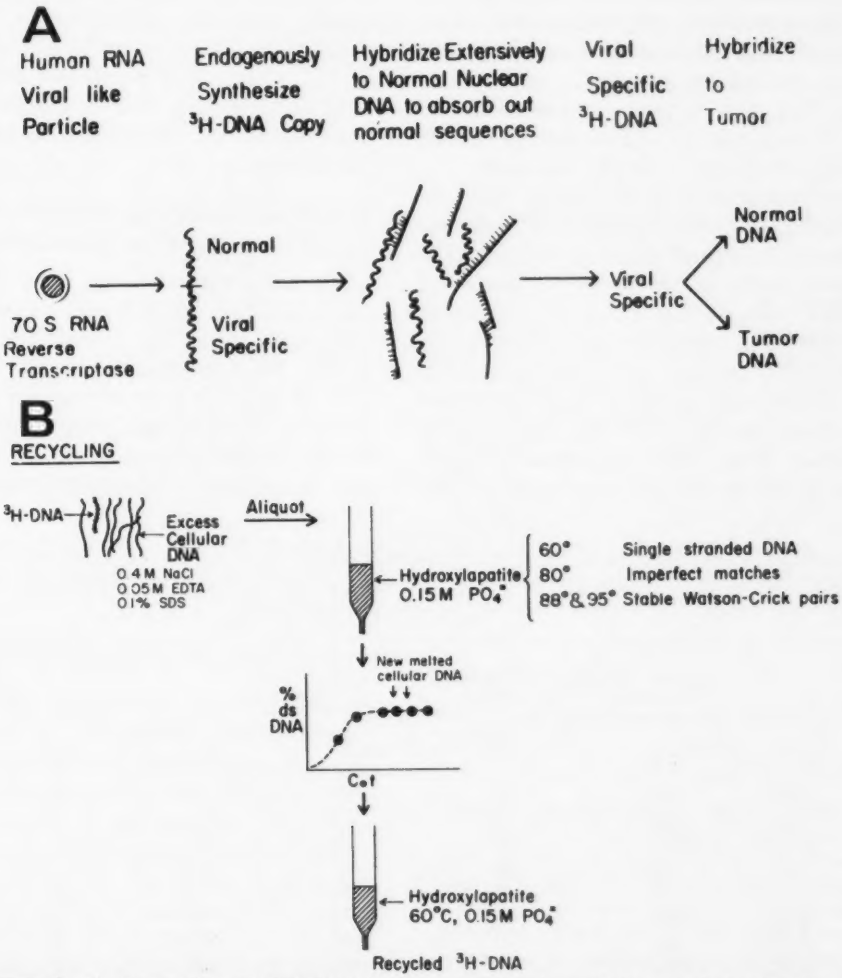

FIGURE 5.—*A*. Generation of [^{3}H]DNA by human leukemic particles and hybridization of sequences shared with normal DNA. *B*. Separation of leukemia-specific sequences by hydroxylapatite chromatography. *See* text for further details.

probe by self-annealing and column chromatography;

d. use the resultant [^{3}H]DNA to detect complementary sequences in normal and leukemic leukocyte DNA;

e. if viral-related sequences are detected in *both*, remove those found in normal leukocytes by exhaustive hybridization to normal DNA; and

f. test the residue for specific hybridizability to leukemic DNA.

In carrying out the recycling and test hybridizations it is imperative that conditions be chosen to account for the possibility that the leukemia-specific sequences are present in only one copy per genome, a possibility that is in fact realized (29). To this purpose, the concentration in moles per liter (C_o) of DNA in nucleotides and the time in seconds (t) of annealing is adjusted to $C_o t$ values of 10,000 that are adequate to locate single sequences.

A typical outcome of hybridizing such recycled tritiated DNA to normal and leukemic DNA is shown in figure 6. It is evident that no complexes stable at temperatures above 88 C are formed with normal DNA. On the other hand, 57% of the recycled [^{3}H]DNA probe forms well-paired duplexes with leukemic DNA. A series of such experiments was performed with particle-generated [^{3}H]DNA and nuclear DNA obtained from eight untreated patients with either acute or chronic myelogenous leukemia. In every case (table 5), the [^{3}H]DNA, after being subjected to exhaustive annealing to normal DNA, yielded a residue that forms stable duplexes only with leukemic DNA. This is in agreement with the experiment in figure 6.

In estimating the implication of this result it must be recalled that the leukemia-specific sequences found (29) in leukemic cells are present as nonreiterated copies per genome. This was established by the $C_o t$ value required to detect them. The sensitivity used to examine normal cells for the leukemia-specific sequences was such that one fiftieth of an equivalent of that found in leukemic cells would have been readily detected. Consequently, one may conclude that the vast majority of normal cells do not contain this particular stretch of malignant information and it cannot therefore be represented in the germ line of nonleukemic individuals.

Unique Sequences in Hodgkin's and Burkitt's Lymphomas and Their Relatedness

We have already seen that like the leukemias, Hodgkin's and Burkitt's lymphomas have particles containing reverse transcriptase and a 70S RNA template related in sequence to that of RLV. It was of obvious interest to determine whether the lymphomas also parallel the leukemias in possessing a unique sequence not detectable in normal tissue. If they do,

TABLE 5.—Exhaustive hybridization of [^{3}H]DNA probe synthesized by leukemic particles with normal-leukocyte nuclear DNA, followed by hybridization of the nonhybridizing recycled leukemic [^{3}H]DNA probe to normal DNA and to leukocyte nuclear DNA from the same leukemic patient (29)*

Patient No.	Disease	Leukemic [^{3}H]DNA hybridized to normal-leukocyte DNA		Recycled leukemic [^{3}H]DNA hybridized to leukocyte DNA			
				Leukemic		Normal	
		Cpm	Hybridization (%)	Cpm	Hybridization (%)	Cpm	Hybridization (%)
1	AML	3020	61	523	56	0	0
2	CML	1350	40	1020	51	0	0
3	CML	2580	51	431	35	3	0
4	CML	510	45	101	36	0	0
5	AML	1100	43	303	48	4	0
6	AML	4520	49	1130	46	1	0
7	AML	390	42	45	69	0	0
8	AML	1450	49	510	52	0	0

*Background was 30 cpm and all counts recorded represent cpm above background. CML = chronic myelogenous leukemia; AML = acute myelogenous leukemia.

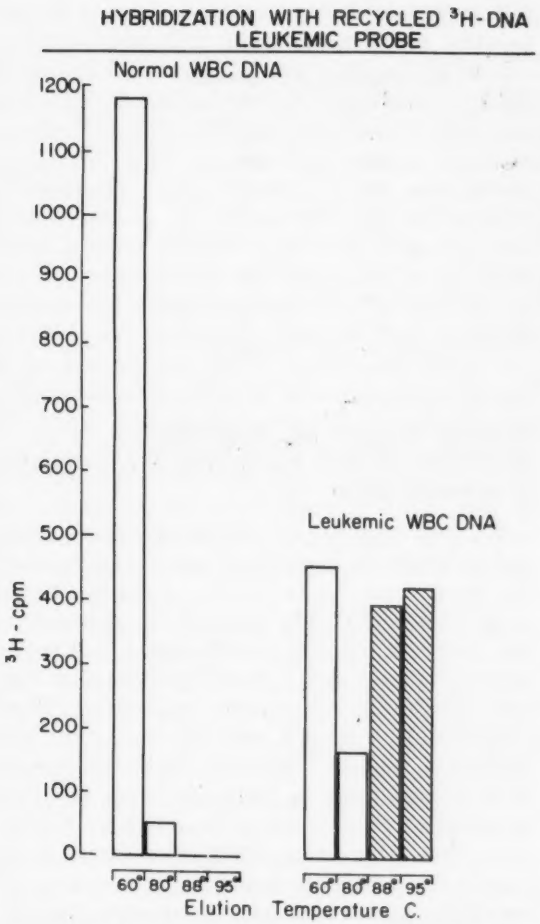

FIGURE 6.—Hydroxylapatite elution profile of a hybridization reaction of recycled leukemic [³H]DNA to nuclear DNA from normal leukocytes and from leukemic leukocytes of the patient from which the [³H]DNA was derived.

one can in addition ascertain whether the sequences found in Hodgkin's and Burkitt's lymphomas are related to each other. The outcome has evident significance for the relevance of the sequence to malignancy.

[³H]DNA probes were synthesized with particles isolated from four Burkitt's tumors and three Hodgkin's disease specimens. Sequences shared with normal DNA (between 35% and 40%) were then removed as described for the leukemias (29) to yield the recycled [³H]DNA probes (30).

Figure 7 shows the outcome of challenging recycled Hodgkin's disease [³H]DNA with nuclear DNA from normal spleen, Hodgkin's disease spleen, and Burkitt's lymphoma. Few, if any, stable duplexes are formed with normal DNA. Note, however, that although the probe was made with Hodgkin's disease particles, the [³H]DNA hybridized to Burkitt's lymphoma nuclear DNA virtually as well as it complexed to DNA from Hodgkin's spleen. The converse is also true, as may be seen from table 6, which summarizes the results of our findings in the recycled [³H]DNA challenged with nuclear DNA from normal and malignant tissues (30). Again, normal DNA is unable to form significant amounts of stable complexes (elution at 88 C and above) with the

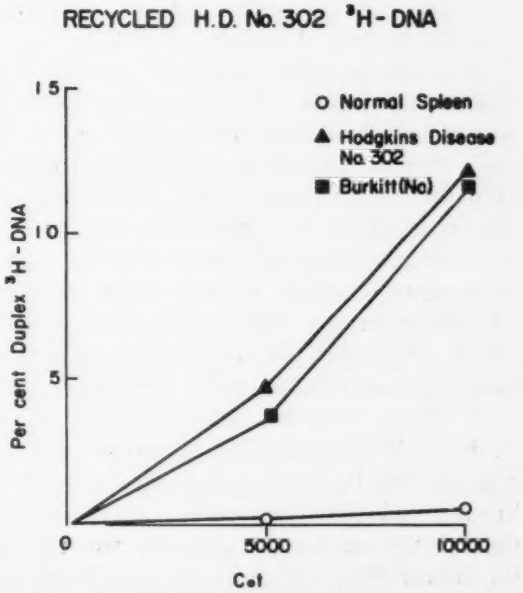

FIGURE 7.—Hybridization of recycled Hodgkin's disease No. 302 [³H]DNA to nuclear DNA isolated from normal spleen (○—○), Hodgkin's disease No. 302 (▲—▲), and Burkitt's lymphoma (Na) (■—■). Equal aliquots were removed from the hybridization vessel at each C_0t value and hybrid formation was analyzed by hydroxylapatite chromatography. The input counts for each point were 1500–2000 cpm and only those duplexes eluting at 88 C and above are counted as stably hybridized.

TABLE 6.—Hybridization of recycled [^{3}H]DNA probes synthesized with human lymphoma particles with nuclear DNA from normal and tumor tissues

Origin of [^{3}H]DNA recycled probe	Nuclear DNA	No. of positives/ total tested	Positives (%)
Burkitt's lymphoma	Burkitt's	7/7	100
	Hodgkin's	2/2	100
	Normal, spleen	0/4	0
	IM,* spleen	0/1	0
	IM,* cell	0/1	0
Hodgkin's disease	Hodgkin's	3/3	100
	Burkitt's	2/3	67
	Normal, spleen	0/3	0

*IM = infectious mononucleosis.

[^{3}H]DNA probes. In all instances, the lymphoma [^{3}H]DNAs hybridized in stable complexes to the nuclear DNA of the original types from which the particles were obtained and used to generate the labeled DNA. Further, with only one exception, all of the Burkitt's and Hodgkin's disease [^{3}H]DNA cross hybridize with each other's DNA.

In summary, several features emerged from this study of the lymphomas. The particle-related sequences found in Burkitt's and Hodgkin's lymphomas possess sequences in common, an observation in accord with our earlier findings (7,13,14,26) that Hodgkin's and Burkitt's particles both share sequences with the murine RLV agent. Further, in view of the previous association of the EBV with Burkitt's lymphoma (9,10) and the nonneoplastic infectious mononucleosis (31,32), it is revealing to note from table 6 that the leukocyte DNA of patients with infectious mononucleosis was devoid of the Burkitt's sequences detected by the recycled [^{3}H]DNA probe, indicating that these latter sequences are specific for neoplastic tissues. The fact that the particle-related sequences in Hodgkin's and Burkitt's tumors are related to each other adds further weight to this conclusion. Finally, the observation that cells carrying multiple copies of the DNA of the EBV do not complex with recycled [^{3}H]-DNA probes from either Hodgkin's or Burkitt's particles proves that these particle sequences have no relation to the DNA of the EBV.

Evidence Against the Chromosomal Inheritance of Viral Information From Studies of Identical Twins

We now come to a biologically more precise and informative experiment on the question of the chromosomal inheritance of the necessary viral information. The comparison of leukemia and lymphoma patients with normals (29,30) suggests that healthy individuals do not contain the neoplastic-specific sequences. However, the data do not rule out the possibility that those who do come down with the disease do so because they in fact inherit the required information in their germ line. One way to resolve this issue is to study the situation in identical twins. This was done in the case of leukemia. Since identical twins are monozygous, ie, derive their genomes from the same fertilized egg, any chromosomally transmitted information must be present in both. If the leukemic member of the pair contains the particle-related DNA sequences, and does so because he inherited them through his germ line, then these same sequences *must* be found in the leukocyte DNA of his healthy sibling. To perform the experiment, it was necessary to locate identical twins with completely convincing evidence for monozygosity and where only one twin was leukemic. Also, the twins had to be of adult age since at least 1 unit of whole blood is required to provide enough leukocyte DNA to carry out the required hybridization.

Two sets of identical twins satisfying all these requirements were found and an experiment similar to the one outlined above was

performed with each pair (33). In each instance, particles containing the reverse transcriptase and 70S RNA were again isolated from the leukocytes of the leukemic members and used to generate the [^{3}H]DNA endogenously. The [^{3}H]DNA was purified and sequences shared with normal DNA were removed by exhaustive hybridization in the presence of a vast excess of normal DNA from random healthy blood donors. This was then followed by hydroxylapatite chromatography to separate paired from unpaired [^{3}H]DNA. It is important to emphasize that in the recycling step, the normal DNA used came from the leukocytes of healthy, random blood donors and not from the normal twin. To have used the latter would have obviously confused the issue. The residue of the tritiated DNA that did not pair with the normal DNA was then used to test for the presence of a sequence in the leukocyte DNA of the patient and that of his healthy sibling.

The results obtained with the two sets of twins are described in figure 8 and it is evident that the same situation occurs between the members of the twin pairs that was observed in the comparison of unrelated leukemic patients and random normals (fig 6 and table 5). The leukemic twin contains particle-related sequences that cannot be detected in the leukocytes of his healthy sibling.

The fact that we could establish a sequence difference between identical twins implies that the additional information found in the DNA of the leukemic members was inserted after zygote formation. This finding argues against the applicability of the virogene hypothesis to this disease since it would demand that the leukemia-specific sequences found in the DNA of the individual with the disease must surely also exist in the genome of his identical twin. These results are also inconsistent with the possibility that individuals who have leukemia have it because they inherit the complete viral genome.

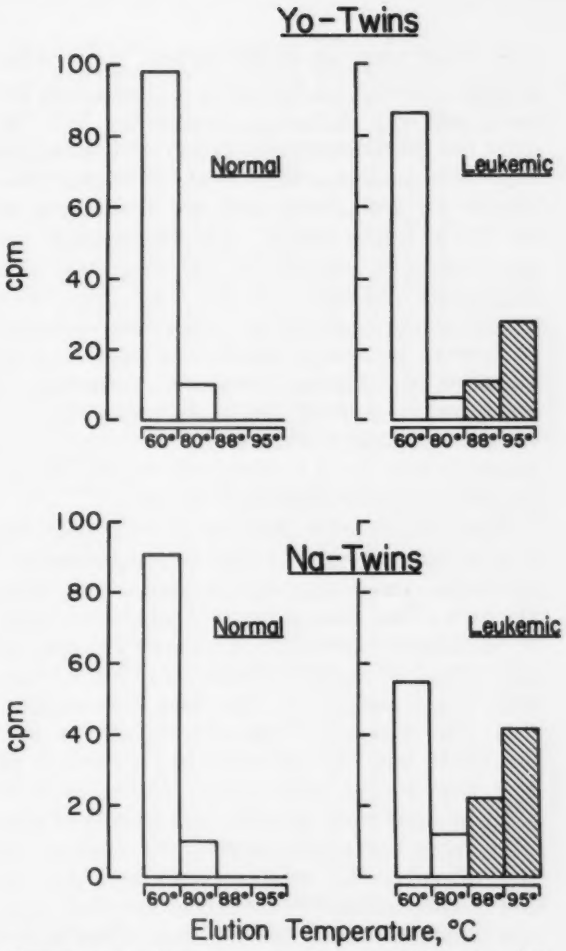

FIGURE 8.—Hydroxylapatite elution profile of a hybridization reaction of the recycled leukemic twin [^{3}H]DNA probe to nuclear DNA from normal leukocytes, normal twin leukocytes, and leukocytes from the same leukemic twin. The annealing reaction mixtures contained 20 A_{260} units of cellular DNA, 0.004 pM of [^{3}H]DNA, and 15 μM of NaH PO_4 (pH 7.2) in a final volume of 0.1 ml. The reaction was brought to 98 C for 60 sec and 0.04 mM of NaCl was added. The reaction mixture was then incubated at 60 C × 50 hrs. The reaction was stopped by the addition of 1 ml of 0.05 M NaH PO_4 (pH 6.8). The sample was then passed over a column of hydroxylapatite of 20-ml-bed volume at 60 C. The column was washed with 40 ml of 0.15 M NaH PO_4 (pH 6.8) at 60, 80, 88, and 95 C. Fractions of 4 ml were collected, the A_{260} of each fraction was read, and the DNA was precipitated with 2 μg/ml of carrier yeast RNA and 10% trichloroacetic acid. The precipitate was collected on Millipore filters, which were dried and counted. In all cases, greater than 80% of the nuclear DNA reannealed. A background count of 8 cpm was subtracted in all instances (33).

Comments

It is important to emphasize that finding unique sequences in the leukemias and lymphomas does not disprove the virogene hypoth-

esis in an absolute sense. Indeed, in a global biologic context, no collection of individual experiments can eliminate hypotheses that invoke the germ-line transmission of a complete copy of some *unspecified* viral information required for malignancy and the production of an RNA tumor virus. The experiments we have described relevant to this issue make this hypothesis unlikely only for those particular neoplasias and associated particulate elements that were in fact subjected to experimental examination. Although not yet achieved, it would not be terribly shocking if some day the virogene concept is established with some neoplasia in man or in a laboratory animal strain inbred for this or some other purpose.

What is evident is that our findings with the human leukemias and lymphomas suggest more optimistic pathways for the control of these diseases. The data suggest that in at least some instances we may not be forced to master the control of our own genes in order to cope with these neoplasias. The data also suggest that the malignant transformation involves the entry and the chromosomal insertion of new viral-related information. Either or both of these steps could provide additional and possibly more accessible targets for chemotherapy. Finally, the existence of the leukemia- and lymphoma-specific sequences and their protein products offers the clinician hitherto unsuspected parameters that could potentially be useful adjuncts in diagnosing and monitoring therapy.

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Orlando, Florida, January 9–11, 1975
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This symposium is sponsored by the Clinical Cancer Investigation Review Committee of the National Cancer Institute. The Program Chairman is Giulio J. D'Angio, MD, Memorial-Sloan Kettering Cancer Center. It is open to physicians interested in cancer therapy; no registration fee will be charged.

Fifth New Drug Seminar

The Division of Cancer Treatment, National Cancer Institute, is planning its Fifth New Drug Seminar to be held on December 16-17, 1974, at the Washington Hilton in Washington, DC. The subject of the seminar will be adriamycin (doxorubicin), a new antitumor antibiotic developed originally in Italy and soon to be commercially available in the United States pending approval by the Food and Drug Administration. Adriamycin is a drug with a wide range of clinical activity. The drug has been found useful in advanced breast cancer, soft tissue and bone sarcomas, lung cancer, pediatric solid tumors, bladder cancer, and malignant lymphomas. The seminar will cover the drug's initial development, mechanism of action, initial clinical evaluation in Italy, as well as a series of detailed reports on the American experience in each of the active tumor types. The seminar should be of interest to oncologists and hematologists, internists, and other physicians with interests in the treatment of cancer. For further information, please contact: Director, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Building 31, Room 3A52, Bethesda, Md 20014.

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